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REDUCTIVE DEHALOGENATION OF POLYBROMINATED AND POLYCHLORINATED BIPHENYLS BY ANAEROBIC MICROORGANISMS FROM SEDIMENT

presented by

Pamela Jo Morris

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Major professor

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REDUCTIVE DEHALOGENATION OF POLYBROMINATED AND POLYCHLORINATED BIPHENYLS BY ANAEROBIC MICROORGANISMS FROM SEDIMENT

By

Pamela Jo Morris

A DISSERTATION

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ABSTRACT

REDUCTIVE DEHALOGENATION OF POLYBROMINATED AND POLYCHLORINATED BIPHENYLS BY ANAEROBIC MICROORGANISMS FROM SEDIMENT

By

Pamela Jo Morris

Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs) are stable industrial chemicals that consist of complex mixtures considered to be highly recalcitrant to biological degradation in the environment. Reductive dehalogenation (when a halogen on the biphenyl molecule is replaced by a hydrogen) is the only known biodegradation process for the more highly halogenated PCB and PBB mixtures. Studies were undertaken to: (1) examine *in situ* reductive debromination in sediments of the Pine River Reservoir, (2) compare the ability of microorganisms from PCB-contaminated and PBB-contaminated sediments to debrominate the commercial PBB mixture, Firemaster, (3) examine factors which might enhance reductive dehalogenation in sediments, (4) evaluate the role of sediment for dechlorinating microorganisms. Sediments in the heavily contaminated region of the Pine River have undergone little or no debromination. Anaerobic microorganisms previously shown to dechlorinate PCB mixtures were unable to dechlorinate Aroclor 1242 in the presence of Pine River sediments located close to the PBB manufacturing site. Microorganisms downstream of the heaviest contamination were able to debrominate Firemaster.

Microorganisms from the Pine River (contaminated with Firemaster), Hudson River (contaminated with Aroclor 1242) and Silver Lake (contaminated with Aroclor 1260), removed 32%, 12%, and 3% of the *meta* plus *para* bromines, respectively, after 32 weeks. The Pine River inoculum removed an average of 1.25 bromines from the biphenyl molecule. When Firemaster was incubated with Hudson River microorganisms (repeatedly transferred with pyruvate and Aroclor 1242), 17% of the *meta* and *para* bromines were removed after 16 weeks, and additional debromination products, 2-bromobiphenyl and biphenyl, were detected. This suggests *ortho* debromination, since all components of Firemaster have at least one *ortho*-substituted bromine. A PCB enrichment culture (with activity over nine transfers) was established using pyruvate as an electron donor and Aroclor 1242 as the electron acceptor. Three Michigan surface soils, Pine River sediments (downstream of the heavily contaminated region), and ashed sediment (with 20% ruminal fluid medium or humic acid) supported reductive dechlorination of Aroclor 1242 by Hudson River microorganisms.

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Introduction

Widespread contamination of the environment by polychlorinated biphenyls (PCBs) has led to increased interest in their environmental fate (Jensen, 1966; Tanabe et al., 1983; Buckley, 1982; Tanabe, 1988). Between 1929 and 1978 approximately 1.4 billion pounds of PCBs were manufactured, and estimates indicate that several hundred million pounds have been released into the environment (Hutzinger and Veerkamp, 1981). In 1972, the E.P.A. issued a report stating that PCB contamination was ubiquitous and an environmental threat (Interdepartmental Task Force on PCBs, 1972). Later, in 1976, Congress passed the Toxic Substances Control Act, which specifically regulated the manufacture, use, and disposal of PCB-contaminated materials (Toxic Substances Control Act, 1976).

Recently, commercial PCB mixtures were shown to undergo reductive dechlorination to yield less chlorinated PCBs (Quensen et al., 1988; Quensen et al., 1990a). Reductive dechlorination of PCBs, in which chlorine is replaced by hydrogen on the aromatic ring, is carried out by anaerobic bacteria from PCB-contaminated sediments. This transformation is important because the dechlorinated products, predominately mono- and dichlorobiphenyls, are less toxic and more susceptible to aerobic degradation. The aerobic and anaerobic biodegradation of PCBs has been recently reviewed (Abramowicz, 1990; Bedard, 1990; Hooper et al., 1990). This anaerobic-aerobic activity offers the possibility for total PCB destruction, and provides a basis for the bioremediation of PCB-contaminated soils and sediments.

While polybrominated biphenyls (PBBs) were manufactured in significantly smaller quantities than PCBs, there is evidence of their widespread environmental contamination (Jansson and Asplund, 1987). While commercial PCB mixtures contain more than 60 congeners, commercial PBB mixtures generally contain fewer

Firemaster (which averages 6 bromines per biphenyl molecule), provides an excellent comparative study for reductive dehalogenation of heavily chlorinated biphenyl mixtures.

This review will focus on the anaerobic-aerobic microbial remediation of polychlorinated and polybrominated biphenyls. Microbial biodegradation offers an alternative to current methods of PCB disposal, including thermal processes (Hunt et al., 1984; Tucker and Carson, 1985) and chemical destruction (Brunelle et al., 1985; Matsunaga et al., 1991).

Manufacturing of Commercial PCB Mixtures

The chlorination of biphenyl in the presence of a catalyst (i.e. iron filings, iron chloride, iodine) produces a mixture of chlorobiphenyls, the exact composition of which is influenced by the ratio of chlorine to biphenyl (Hutzinger et al., 1974). Chlorination of biphenyl can produce 209 possible chlorobiphenyls (PCB congeners) substituted with 1 to 10 chlorine atoms. The chlorinated biphenyls were manufactured and sold as complex mixtures, which differed in the percent chlorine by weight, and ranged from being oily liquids to sticky resins (Table 1). The PCB mixtures have been manufactured under various trade names, including Aroclor (Monsanto, U.S.); Phenoclor and Pyralene (Prodelec S.A., France); Clophen (Farbenfabriken Bayer, AG. Germany); and Kanechlor (Kanegafuchi Chemical Industrial Co. Ltd, Japan). The commercial PCB mixtures manufactured in the United States, Aroclors, were distinquished by a four digit number. The first two digits indicate the number of carbon atoms, while the last two digits indicating the degree of chlorination. For example, Aroclor 1260 contains 12 carbon atoms, and is 60% chlorine by weight.

Table 1. Physical properties of the commercial PCB mixtures, Aroclors.

Aroclor	% Cl	Water Solubility	Avg. No. Cl/Biphenyl	Avg. Mol. Wt.	Physical State at Room Temp.
1221	21	-	1.15	192	mobile oil
1242	42	200	3.10	261	mobile oil
1248	48	100	3.90	288	mobile oil
1254	54	40	4.96	327	viscous liquid
1260	60	25	6.30	372	sticky resin

Physical Properties of PCB Mixtures

The commercial PCB mixtures are known for their thermal stability, resistance to oxidation, acids, bases, and other chemicals, and their excellent dielectric properties (Hutzinger et al., 1974). These physical properties led to their use as dielectric fluids (capacitors, transformers), industrial fluids (hydraulic systems, gas turbines, vacuum pumps), fire retardants, and plasticizers (adhesives, textiles, surface coatings, sealants, printing, carbonless reproducing paper). The PCB mixtures have low solubility in water, and solubility decreases with an increase in the degree of chlorination (Table 1).

Anaerobic Reductive Dechlorination of PCBs

Reductive dechlorination of PCBs occurs when a chlorine on the biphenyl molecule is replaced by a hydrogen. In general, the products of dehalogenation of aromatic compounds are less toxic and more susceptible to further degradative processes. Since it was first reported by Suflita et al. (1982), reductive dehalogenation involving aromatic C-Cl bonds has been observed within a number of anaerobic microbial communities, i.e. enrichments from sewage sludge (Boyd et al., 1983; Mikesell and Boyd, 1985; Fathepure et al.,1988), pond sediment (Gibson and Suflita, 1986; Struijs and Rogers, 1989), and aquifers (Kuhn and Suflita, 1989). A strict anaerobe capable of reductive dehalogenation of *meta*-substituted chlorobenzoates, *Desulfomonile tiedjei*, has been isolated in pure culture from municipal sewage sludge (Shelton and Tiedje, 1984; DeWeerd et al., 1990). Currently, there is no microorganism in pure culture capable of reductive dechlorination of PCBs, and the PCB dechlorination activities described to date are derived from one or more members of mixed communities eluted from PCB-contaminated sediments.

Although the communities involved in PCB dechlorination have not been elucidated, there is speculation to what role PCBs might play in an anaerobic system. In anaerobic sediments, PCBs may act as an alternative electron acceptor, and this may be important in environments where electron acceptors (e.g. sulfate, carbon dioxide) are often limiting. Additionally, the dechlorination of PCBs might yield energy for the microorganisms involved in the reaction. Reductive dechlorination of *meta*-substituted chlorobenzoates by *Desulfomonile tiedjei* has been shown to yield energy for growth (Mohn and Tiedje, 1990; Dolfing, 1990).

Environmental Reductive Dechlorination of PCBs

Capacitor manufacturing plants at Hudson Falls and Fort Edward, New York, released Aroclor 1242 into the Hudson River between 1952 and 1971. Altered PCB patterns observed subsequently in Hudson River sediment samples suggested the occurrence of *in situ* microbial reductive dechlorination (Brown et al., 1987a). The general observation apparent in gas chromatographs of these sediments was the depletion of heavily chlorinated congeners with concomitant increases in the concentrations of certain lower chlorinated PCB congeners. Alterations in the GC patterns from PCB-contaminated sediments of the upper Hudson River represented 4 distinct patterns, designated A, B, B', and C (Brown et al., 1987a). Pattern A was of unaltered Aroclor 1242 and occurred in Hudson River sediment surface deposits. Patterns B, B', and C were found in subsurface sediments, and showed lower levels of tri-, tetra-, and pentachlorobiphenyls, and increased levels of mono- and dichlorobiphenyls. Sediments analyzed from a number of locations (upper and lower Hudson River, NY; Silver Lake, Pittsfield, MA; Waukegon Harbor, IL; Sheboygan Harbor, WI; New Bedford Harbor, MA; Escambia Bay, Pensacola, FL; the Acushnet Estuary, New Bedford, MA; the Housatonic River, CN; and the Hoosic River, North Adams, MA) exhibited similar

congener distribution patterns (plus new patterns) suggestive of reductive dechlorination (Brown et al., 1984; 1987a; 1987b; 1988; 1990). Generally, the environmental PCB congener distribution patterns result from the selective removal of *meta* and *para* chlorines, although examples of sediments showing *ortho*, *meta*, and *para* dechlorination have also been reported. This selective removal of chlorines suggests that different enzymes, and different microorganisms are responsible for the observed activities.

Laboratory Studies

The suggestion by Brown et al. (1987a) that altered PCB congener profiles of *in situ* PCB contaminated sediments were due to reductive dechlorination was verified in the laboratory (Quensen et al., 1988), when microorganisms from PCB-contaminated Hudson River sediments were found to reductively dechlorinate many of the congeners in Aroclor 1242. Since that report, a number of laboratory studies have demonstrated reductive dechlorination of PCBs by anaerobic microbial communities (Quensen et al., 1990a; Alder et al., 1990; Nies and Vogel, 1990; Abramowicz et al., 1991; Van Dort and Bedard, 1991).

Quensen et al. (1988) developed a transfer technique wherein a reduced anaerobic mineral medium (RAMM) was used to elute anaerobic bacteria from Hudson River sediment which were then used to inoculate non-PCB contaminated upstream sediments that had been spiked with Aroclor 1242. For the preincubation, anaerobic microorganisms eluted from non-PCB contaminated Hudson River sediment were added to non-PCB-contaminated Hudson River sediments, and preincubated until methane was detected. This preincubation period was used to consume all residual O_2, and assure strict anaerobic conditions. Next, the preincubated cultures are autoclaved. Anaerobic microorganisms eluted from PCB-contaminated Hudson River sediments were used to inoculate the autoclaved

sediments and were amended with known concentrations of commercial Aroclors. The advantage of this technique was that the detection of dechlorination products was not confounded by peaks from PCB-contaminated sediment that had previously undergone dechlorination. The only carbon source available was supplied by the sediment, i.e., no external carbon source was added.

Using this experimental approach, Quensen et al. (1988) observed a 53% decrease in the total chlorine from 700 ppm Aroclor 1242 in 16 weeks, resulting in an increase in the proportion of mono- and dichlorobiphenyls. In this study, dechlorination occurred primarily from the *meta* and *para* positions, resulting in an accumulation of *ortho*-substituted congeners. Less dechlorination was observed at 140 ppm Aroclor 1242, and no measurable dechlorination observed at 14 ppm. There are two possible explanations for the observed concentration dependence. A higher concentration in the sediment should result in higher solution concentrations (Chiou et al., 1979), and only PCBs in solution may be available for microbial uptake (Ogram et al., 1985). Also, specific congeners in the mixture may need to be in a high enough concentration to induce dechlorination activity.

In a subsequent study, Quensen et al. (1990a) evaluated the reductive dechlorination of Aroclors 1242, 1248, 1254, and 1260. Microorganisms from an Aroclor 1242-contaminated site (upper Hudson River, NY) dechlorinated Aroclor 1242 to a greater extent than did microorganisms from an Aroclor 1260-contaminated site (Silver Lake, MA), which dechlorinated Aroclor 1260 more rapidly than Aroclor 1242 (Quensen et al., 1990a). Maximal observed dechlorination rates by Hudson River microorganisms were 0.3, 0.3, and 0.2 μg-atoms of chlorine removed per g of sediment per week for Aroclors 1242, 1248, and 1254, respectively. The maximal observed dechlorination rates for Hudson River and Silver Lake microorganisms for Aroclor 1260 were 0.04 and 0.21 μg-atoms of chlorine removed per g of sediment per week, respectively. The rate of dechlorination tended to

decrease as the degree of chlorination of the Aroclor increased, especially for Aroclor 1260 (which averages approximately 6 chlorines per biphenyl molecule). Again, there was a preferential loss of chlorine from the *meta* and *para* positions of the biphenyl molecule.

There is one report of *ortho* dechlorination in laboratory studies. In this study, the relative molar distribution of 2,3,5,6-chlorobiphenyl (or 2,3,5,6-CB) and its dechlorination products were monitored over time (Van Dort and Bedard, 1991). The major dechlorination products were 2,3,6-CB (16%), 2,6-CB (63%), and 2,5-CB (21%) after 37 weeks of incubation. This study suggests the possibility of obtaining anaerobic microorganisms capable of dechlorinating PCBs down to biphenyl. However, the lengthy incubation periods will make enrichment of *ortho* dechlorination activity very difficult.

Characterization and Optimization of PCB Dechlorination Activity

While anaerobic dechlorination of PCBs can now be readily demonstrated in laboratory studies to be microbiologically-mediated, characterization and optimization of this activity has proven more difficult. The enhancement of anaerobic dechlorination in laboratory incubations from the addition of a mineral medium (specifically the metals) or detergents (e.g., Triton X-705) to sediment slurries has been observed (Abramowicz et al., 1991).

The effect of acetate, acetone, methanol, or glucose on the anaerobic reductive dechlorination of Aroclor 1242 has been examined (Nies and Vogel, 1990). Dechlorination was weakly stimulated by methanol, glucose, and acetone when added to PCB-contaminated Hudson River sediments (containing approximately 20 ppm Aroclor 1242) to which 300 ppm of new Aroclor 1242 was added. The PCB dechlorination pattern was similar regardless of which organic substrate was added. In this study, no significant dechlorination in incubations

receiving no additional organic substrate (other than that provided by sediment) was observed, i.e., the addition of an organic substrate not only accelerated dechlorination but was also required for dechlorination activity. This was a different result than from the work by Quensen et al. (1988; 1990a) where an added carbon source (other than that provided by the sediment) was not required for dechlorination activity.

In the transfer technique by Quensen et al. (1988; 1990a), non-PCB-contaminated Hudson River sediment was preincubated with microorganisms eluted from non-PCB-contaminated Hudson River sediments until methane was detected, and then the sediments were autoclaved prior to inoculation and addition of the Aroclors. This preincubation step adds carbon to the microbial community, resulting in a removal of residual O_2. Also, autoclaving probably resulted in a release of carbon and nutrients to the anaerobic community. In the study of Nies and Vogel (1990), available carbon could have been limited. In Nies and Vogel (1990), 49% of the total chlorine in Aroclor 1242 was removed to produce mono- and dichlorobiphenyls, compared to 90% of the total chlorine in Quensen et al. (1988).

Previous studies on reductive dechlorination of PCBs have been dependent on PCB-contaminated sediment as an inocula source. The addition of pyruvate to the culture medium improved the transferability of Aroclor 1242-dechlorinating cultures on non-PCB contaminated sediments compared to that of mineral medium alone. Dechlorination activity was maintained for 9 serial transfers on non-PCB contaminated Hudson River sediment (non-sterile) for one year (Morris, this publication, Chapter 4). In this study, cultures were not preincubated until methane production was detected, and an initial acclimation period of about 12 weeks was decreased to four weeks in succeeding transfers with pyruvate. Predominately *meta* chlorines were removed, suggesting loss of *para* dechlorination activity or

enrichment for *meta* dechlorination over time. Interestingly, clean Hudson River sediment (air-dried) with pyruvate-amended mineral medium (with no inoculum added other than that supplied by the sediment) also showed dechlorination activity after an acclimation period of approximately 20 weeks. Dechlorination has also been observed by microorganisms from a river sediment with no historical exposure to PCBs (Nies, personal communication). This implies that the PCB dechlorinating activity may be a more widespread activity than previously suggested.

Anaerobic Dechlorination: Solid Support Requirement

All anaerobic studies on reductive dechlorination of PCBs have required a solid support. This requirement has made characterization and optimization of PCB dechlorination activity more difficult. Most of the previous studies have been conducted in the presence of non-PCB contaminated Hudson River sediments, located upstream from the site of contamination (Quensen et al., 1988, 1990a; Morris, this publication, Chapter 4), or PCB-contaminated sediments (Nies and Vogel, 1990). It is currently unknown whether the sediment supplies an important nutrient/carbon source to the dechlorinating microorganisms, or influences the bioavailability of the PCBs.

Griffith et al. (1990) studied the dechlorination of Aroclor 1242 by Hudson River microorganisms in the presence of various organic and inorganic substrata, including quartz sand, vermiculite, sawdust, peat, smectite clay, and 20%/80% mixtures of peat with sand, vermiculite, or clay. Vermiculite, clay, sand, or sawdust alone did not support dechlorination in 40 weeks of incubation. The peat/vermiculite treatment supported dechlorination activity almost as well as Hudson River sediments, removing an average of 1.2 chlorines from Aroclor 1242 after 25 weeks of incubation. The presence of different substrata influenced not only the extent of dechlorination, but also whether dechlorination was observed

primarily from the *meta* or *para* positions (or both). Thus, the substrata may be influencing which microbial members of the dechlorinating community are enriched.

The use of Hudson River sediments which had been ashed to remove organic carbon did not support dechlorination (Morris, this publication, Chapter 5). The addition of ruminal fluid or Aldrich humic acid to the medium with the ashed sediments supported only half the dechlorination activity compared to that of Hudson River sediments. Interestingly, ruminal fluid-amended medium and humic acid alone did not support dechlorination.

Anaerobic Mineralization

The anaerobic dechlorination studies discussed above have observed only chlorine removal, while the biphenyl nucleus remains intact. Tiedje et al. (1991) added biphenyl, 2-CB, 2,2'-CB, or 2,6-CB to methanogenic sediment slurries inoculated with Hudson River microorganisms. No evidence of dechlorination or degradation of these compounds was observed during one year of incubation. These three congeners are common end products of the anaerobic dechlorination of Aroclor 1242. This process is different from processes that may result in the mineralization of PCBs, which has been recently reported (Rhee et al., 1989; Chen et al., 1988). This study investigated the anaerobic biodegradation of Aroclor 1221 by biphenyl-grown Hudson River microorganisms, and observed 53% loss of mainly mono- to pentachlorobiphenyls under a N_2 atmosphere, but not under a CO_2/H_2 atmosphere. Additionally, under a N_2 atmosphere, methane was undetectable, which contrasts with the methanogenic conditions observed during anaerobic reductive dechlorination. This suggest that under anaerobic conditions, the lesser chlorinated PCBs were being mineralized, implying an attack on the biphenyl ring, although no metabolites were identified. The authors did not observe the

accumulation of less-chlorinated congeners as a result of reductive dehalogenation under anaerobic conditions. More extensive studies on the possibility of anaerobic mineralization need to be conducted, with highly controlled analytical tests.

Aerobic Degradation of PCBs

In the earliest reported isolation of biphenyl and 4-chlorobiphenyl-degrading strains (*Achromobacter* sp.), Ahmed and Focht (1973) identified both the *meta*-cleavage product and *p*-chlorobenzoic acid as metabolites of the degradation pathway. Since this report, a number of pure cultures of microorganisms, as well as naturally occurring microbial communities, have been found to be capable of degrading PCBs. Furukawa and Matsumura (1976) later isolated from lake sediment an *Alkaligenes* sp. capable of metabolizing a variety of PCBs, with preferential degradation of the less chlorinated ring. The aerobic degradation of PCBs has been extensively reviewed (Furukawa, 1982; Furukawa, 1986; Abramowicz, 1990). Aerobic degradation rates of PCBs are enhanced by the addition of biphenyl as the carbon source (Brunner et al., 1985; Focht and Brunner, 1985). Biphenyl (Gibson et al., 1973), monochlorobiphenyls (Kong and Sayler, 1983; Masse et al., 1984; Shiaris and Sayler, 1982; Shields et al., 1985) and sometimes dichlorobiphenyls (Adriaens et al., 1989) have been shown to serve as sole carbon sources, or be cometabolized when grown on biphenyl by a number of pure and mixed cultures of microorganisms.

Bedard et al. (1986) isolated natural aerobic bacteria capable of degrading PCBs in nearly every contaminated soil and sediment tested. Cultures were enriched on biphenyl as the sole carbon and energy source. Using this approach, a diverse group of 25 strains of PCB degrading bacteria were isolated and characterized. All organisms isolated were capable of degrading the lightly chlorinated PCBs. In general, degradation of PCBs usually involves initial addition

of O_2 at the 2,3-position by a dioxygenase enzyme, followed by dehydrogenation to the catechol and ring cleavage. The less chlorinated ring is preferentially degraded. This pathway is similar to the degradation pathways for biphenyl (Gibson et al., 1973) and toluene (Finette et al., 1984).

PCBs may also be metabolized through other routes. For example, 4-, 2,3-, or 3,4-substituted rings are more readily metabolized by *Corynebacterium* MB1 (employing a 2,3-dioxygenase) while 2-, 2,4-, 2,5-, and 2,4,5-substituted rings are more readily metabolized by *Alcaligenes eutrophus* H850. This has led to the proposal that a significant mechanism for PCB metabolism in these organisms involves a novel 3,4-dioxygenase attack (Bedard et al., 1987a). It is clear that the congener specificity indicates two distinct classes of dioxygenases. The 3,4-dioxygenase occurs in *Acinetobacter* P6, in *Alcaligenes eutrophus* H850 and *Pseudomonas putida* LB400. These two distinct types of dioxygenases tend to be complementary and allow a broader range of PCB congeners to be degraded.

Aerobic Degradation of Highly Chlorinated PCBs

More highly chlorinated PCB congeners have not been shown to serve as growth substrates, and aerobic degradability generally decreases with increasing chlorine number (Furukawa et al., 1978b; Bedard, 1990). Higher chlorinated levels may result in hinderance of the 2,3-dioxygenase by chlorine substitution at either of these two positions. However, studies have been reported on the bacterial transformation of Aroclor 1254 (Sayler et al., 1977; Bedard et al., 1987b; Kohler et al., 1988). Resting cells of *Alcaligenes eutrophus* H850 have been shown to degrade components of 21 out of 44 capillary peaks, resulting in degradation of 35% of Aroclor 1254 (Bedard et al., 1987b). Several aerobic bacterial strains have been shown to degrade a large range of congeners. These include *Pseudomonas* strain LB400 (Bopp,1986), *Alcaligenes eutrophus* H850 (Bedard et al., 1987a and 1987b),

Corynebacterium strain MB1 (Bedard et al., 1987a and 1987b), and *Acinetobacter* strain P6 (Furukawa et al., 1978a; Furukawa et al., 1979).

Genetic Engineering

Until recently, little was known about the genes that encode the enzymes responsible for PCB degradation. The genes encoding bacterial degradation of PCBs have been isolated and utilized to construct recombinant organisms capable of degrading PCBs. The genes encoding the PCB degradation pathway have been cloned from *Pseudomonas* sp. LB400, *Alcaligenes eutrophus* H850, and *Pseudomonas putida* OU83 (Mondello, 1989). A recombinant *E. coli* containing genes from *Pseudomonas* sp. LB400 has been shown to degrade Aroclor 1242 and did not require growth on biphenyl to achieve high levels of degradative activity unlike the donor strain (Mondello, 1989).

Yates and Mondello (1989) used DNA-DNA hybridization to compare *Pseudomonas* strain LB400 for PCB degradation with seven other PCB-degrading strains. Significant hybridization was detected to the genome of *Alcaligenes eutrophus* H850, while DNA from the other PCB-degrading strains (e.g., *Corynebacterium* sp. MB1) showed no hybridization to the probe, implying the existence of at least two distinct classes of genes encoding PCB degradation. The authors suggest that these genes may have been acquired through DNA transfer within bacterial populations in the environment.

Anaerobic-Aerobic PCB Degradation

The anaerobic reductive dechlorination of Aroclor 1242, which averages roughly three chlorines per biphenyl molecule, results mainly in mono- and dichlorobiphenyls, i.e. 2-CB, 2,2'-CB and/or 2,6-CB (coeluting isomers), and some trichlorobiphenyl, i.e., 2,2',6-CB. Roughly 90% of the PCBs resulting from reductive

dechlorination of Aroclor 1242 are *ortho*-substituted. This partial transformation of Aroclor 1242 results in the removal of approximately half of the total chlorines. The benefit of this partial transformation is that the anaerobic products are susceptible to furthur degradation by aerobic microorganisms. Additionally, there is a toxicity reduction of Aroclor 1242 due to the preferential removal of *meta* and/or *para* chlorines (Quensen et al., 1990b). The coplanar PCB congeners (which exhibit the greatest dioxin-like toxicity) in Aroclor 1242 decreased by 85 to 96% during a 16 week incubation with microorganisms eluted from Hudson River sediments, and showed a 75% toxicity reduction by an ethoxy resorufin *O*-deethylase induction assay (Tillet et al., 1991).

Polybrominated Biphenyls

Polybrominated biphenyls are a group of industrial compounds used as flame retardants in synthetic polymers (e.g. polyacrylonitrile, polybutadiene, and polystyrene). The commercial production of PBBs began in 1970, and approximately 13.3 million pounds of PBBs were produced in the U.S. between 1970 and 1976 (Di Carlo et al., 1978). While they are similar structurally to PCBs, little was known about these compounds until in 1973 when 500 to 1,000 pounds of the flame retardant was accidentally used instead of the dairy feed additive magnesium oxide, resulting in widespread contamination of animal feeds, animals, soils, and human food products (Carter, 1976; Fries, 1985).

Polybrominated biphenyls were manufactured in the U.S. into mixtures which differed in the extent and positions of bromination, analagous to commercial PCB mixtures. Unlike PCBs which are liquids, PBB mixtures are solids, and their solubility in water has been estimated as 11 ppb to 610 ppb (Di Carlo et al., 1978). Solubility decreases with increasing bromination. The Michigan Chemical Corp. (St. Louis, MI) produced a PBB mixture composed primarily of hexabromobiphenyl

(Firemaster), and White Chemical Co. (Bayonne, N.J.) and Hexcel Corp. (Sayreville, N.J.) produced predominately octabromobiphenyl and decabromobiphenyl mixtures. Only 40 of the 209 possible PBB congeners have been synthesized into pure form.

Environmental losses of PBBs at its sites of manufacture have been estimated at 51,000 lb / million lb of product produced through emission to air from vents of the hydrogen bromide recovery system, losses in waste waters, and solid losses to landfills (Di Carlo et al., 1978). Photolysis of PBBs containing 2 to 8 bromine atoms shows that bromines are removed most readily from the *ortho* position (Ruzo et al., 1976). Changes in PBBs in surface soils have been attributed to photochemical decomposition (Hill et al., 1982), and other studies have demonstrated the lack of microbial degradation of PBBs in contaminated soils (Jacobs et al., 1976).

The Pine River (St. Louis, MI) is a site in which a commercial PBB mixture, Firemaster, is a known sediment contaminant. Along with the PBB loadings, this site is also contaminated with petroleum, hexabromobenzene, chlordane, DDT, and heavy metals (Forba, 1980; Morris, this publication, Chapter 2)). Firemaster is a less complex mixture than the Aroclors, with 9 congeners being the predominant components of the mixture, in contrast with 60 to 80 congeners in the Aroclor mixtures. One congener, 2,4,5-2',4',5'-hexabromobiphenyl comprises over 50% of the PBB mixture (Sundstrom et al., 1976).

Until recently, little was known about the fate of Firemaster in anaerobic environments such as sediments. This author has demonstrated that anaerobic microorganisms eluted from three different river sediments are capable of reductive debromination of Firemaster in laboratory studies (Morris, this publication, Chapter 3). Anaerobic microorganisms eluted from sediments of the Pine River (St. Louis, MI), Hudson River (Hudson Falls, NY), and Silver Lake (Pittsfield, MA) removed

32%, 16%, and 5% of the *meta* plus *para* bromines, respectively, after 32 weeks of incubation. In general, there was a 20 week lag prior to measurable debromination activity, which is similar to results of dechlorination of Aroclor 1260. Both Firemaster and Aroclor 1260 average six halogens per molecule. The more heavily chlorinated mixtures have been the most difficult to dechlorinate, and no aerobic degradation activity has been observed with Aroclor 1260. Therefore, the ability to first remove halogens from the heavily halogenated mixtures is a critical first step in their bioremediation.

Summary

Reductive dechlorination of polychlorinated biphenyls, while suggested in environmental samples, has been verified in the laboratory to be mediated by anaerobic microorganisms eluted from sediments. Reductive dechlorination generally results in removal of *meta*- and *para*-substituted chlorines, while *ortho*-substituted chlorines appear more resistant to removal. Reductive dechlorination of commercial PCB mixtures, Aroclors, generally results in the accumulation of mono- and di-substituted chlorobiphenyls. The products of reductive dechlorination of Aroclors are susceptible to aerobic degradation by a number of mixed microbial communities as well as by pure cultures of microorganisms. The commercial PBB mixtures, which were manufactured in significantly smaller quantities than PCBs, are less well studied. However, PBBs have been shown to undergo reductive dehalogenation under anaerobic conditions by microorganisms from PBB and PCB-contaminated sediments.

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Chapter 2:

An Assessment of the Reductive Debromination of Polybrominated Biphenyls in the Pine River Reservoir

Introduction

Polybrominated biphenyls (PBBs) were manufactured as fire retardants for thermoplastic applications under the tradename of Firemaster by Michigan Chemical Corporation (later Velsicol Chemical Corporation) between 1970 and 1974 (Di Carlo et al., 1978; Fries, 1984). Production of the PBB flame retardant in Michigan totalled approximately 11 million lb between 1971 and 1974 (Hesse and Powers, 1978). Concern over the environmental and health effects of PBBs were raised following an incident in 1973 when between 500 and 1,000 pounds of the flame-retardant were accidentally substituted for the dairy feed additive magnesium oxide. This resulted in widespread contamination of animal feeds, animals, soils, sediments, and human food products (Carter, 1976; Forba, 1980; Forba, 1982; Fries, 1984). The Pine River Reservoir at St. Louis, Michigan, adjacent to the former Michigan Chemical Corporation plant, is contaminated with PBBs, as well as with petroleum, hexabromobenzene, chlordane, DDT, and heavy metals (Forba, 1980; Forba, 1982). It has been estimated that 56,000 lb of DDT, 10,000 lb of HBB, and 800 lb of PBB are contained in Pine River Reservoir sediments. The maximum concentration of Firemaster found was 300 μg/g, with an average concentration of 8.6 μg/g PBBs (Forba, 1980; Forba, 1982).

The PBB mixture is chemically and toxicologically related to polychlorinated biphenyls (PCBs) (De Voogt and Brinkman, 1989). However, while the PCB mixtures (Aroclors) contain up to 80 congeners, Firemaster contains only 9 predominant congeners. Approximately 50% of the mixture is composed of a single congener, 2,4,5-2',4',5'-hexabromobiphenyl (Sundstrom et al., 1976). Like PCBs, PBBs are considered to be persistent in most natural environments (Filonow et al., 1976; Jacobs et al., 1976; Jacobs et al., 1978; Jansson and Asplund, 1987). However, congener distribution patterns of PCBs in sediments of the Hudson River (near Hudson Falls, NY) and at other sites suggested that the PCBs have undergone

microbial reductive dechlorination (Brown et al., 1984; Brown et al., 1987a; Brown et al., 1987b; Brown and Wagner, 1990). This was confirmed in the laboratory when microorganisms eluted from PCB-contaminated Hudson River sediments were shown to dechlorinate commercial PCB mixtures, Aroclors (Quensen et al., 1988; Quensen et al., 1990). In these studies, chlorines were removed from the *meta* and *para* positions of the biphenyl, while *ortho* chlorines were not removed. This previously unrecognized environmental transformation of PCBs is important because the dechlorinated products are less toxic and more susceptible to further biodegradation including destruction of the biphenyl rings.

The primary purpose of this study was to determine if reductive debromination of PBBs has occurred in sediments of the Pine River by conducting congener specific analysis of the PBBs present in the sediments. The potential for anaerobic dehalogenation to occur in Pine River sediments was evaluated by assaying for the presence of PCB and PBB dehalogenating microorganisms, and for the ability of Pine River sediments to support PCB and PBB dehalogenation. Sediment characteristics that might influence *in situ* reductive dehalogenation were also examined.

Materials and Methods

Sediment sampling and analyses. Aroclor 1242-contaminated sediments were collected (August 1988) from the upper Hudson River at River Mile 193.5. Non-PCB contaminated Hudson River sediments (Hudson Falls, NY) were collected further upstream at River Mile 205. Hudson River sediments were collected with a post hole digger to a depth of approximately 25 cm and then transported to the laboratory in full, sealed paint cans. Sediment cores (Cores 1, 2, and 3) from the Pine River reservoir (St. Louis, MI) were collected in January of 1987 and 1988 (refer to Figure 1 for sampling locations). Pine River sediments were

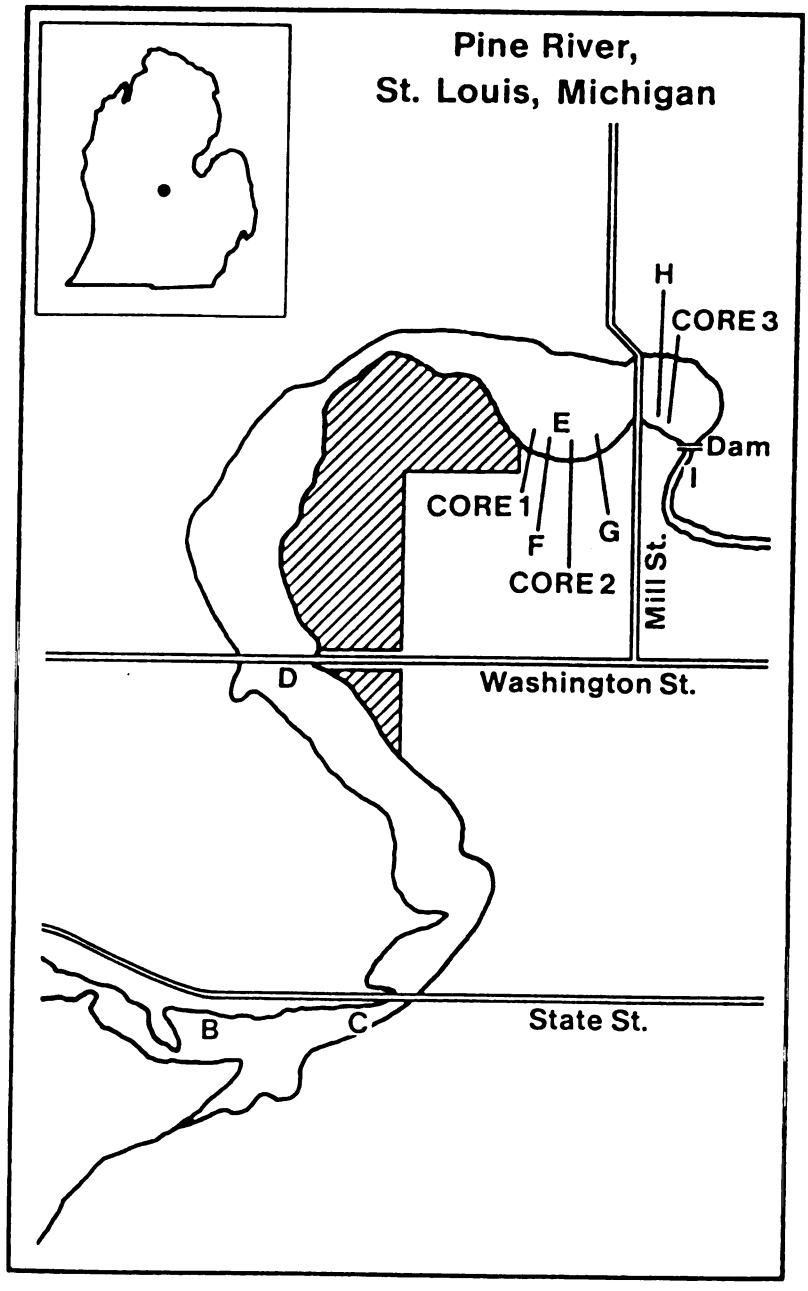

FIGURE 1. Sediment sampling locations from the Pine River, St. Louis, Michigan. Sediment cores 1, 2, and 3 were analyzed for congener-specific analysis of Firemaster and potential debromination products. Sediment samples B (clean Pine River sediment), C, D, and E (the site of highest co-contamination) were used to study the ability of these sediments to support Aroclor 1242-degrading microorganisms from Hudson River sediments. Sediments E, G, H, and I were used to elute microorganisms to assay for reductive dehalogenation of PBBs and PCBs, and contain varying concentrations of PBBs. The shaded area represents the PBB manufacturing site, Michigan Chemical Company (later Velsicol Chemical Corporation).

collected by pushing a 3 inch PVC pipe into the sediments to approximately 25 cm. To retrieve the sediment, the pipe was plugged witha rubber stopper and withdrawn. The pipe was then sawed at the sediment-water interface and then capped for transporation to the laboratory. Pine River sediment grab samples were taken in February of 1988 (refer to Figure 1 for sampling locations B through I) for PBB and PCB dehalogenation assays. Sediment samples B, C, D and E were used for studying the ability of Pine River sediments to support an anaerobic PCB-dechlorinating culture from the Hudson River. Sediment samples F, G, H and I were used to elute microorganisms which were assayed for their ability to dehalogenate PBBs and PCBs.

Sediment cores were stored at -20^{0}C until extraction and analysis. When removed from storage, cores were sectioned into 2 cm sections and air-dried. Two g subsamples from each 2 cm section were added to prerinsed cellulose thimbles, and Soxhlett extracted with hexane/acetone (9:1) for 24 h. The organic extract was then shaken overnight for 24 h with elemental mercury to remove sulfur, since this compound interferes with electron capture detection. This hexane/acetone extract was treated with concentrated H_2SO_4, and then two 2% NaCl rinses. Finally, the sample was dried with Na_2SO_4, and passed through columns containing acid-rinsed copper filings and Florisil. The final sample volume was then adjusted by evaporation or addition of hexane prior to analysis by capillary gas chromatography.

Sediment characterization. Oil and grease content was determined as described by Boyd and Sun (1989). Essentially this procedure involved mixing 20 g of soil (acidified to pH 2 with concentrated HCl) and 10 g of $MgSO_4$. After 15 min, the sample was extracted with 200 ml of 1,1,2-trichlorotrifluoroethane in a Soxhlet apparatus for 24 h. The oil and grease remaining was determined gravimetrically. Organic carbon (after oil and grease extraction) was determined as CO_2 released from combustion minus CO_2 released upon acidification (analysis by Huffman

Laboratories, Inc., Golden, Colorado). Particle size analysis was determined by the Michigan State University Soil Testing Laboratory (East Lansing, MI) using the hydrometer method (Gee and Bauder, 1986). Sulfate concentration in air-dried sediments was measured by mixing 5 g of sediment with 50 ml of 0.15% $CaCl_2$, shaking for 30 min., and filtering (Whatman no. 42 filter paper). The resulting aqueous extract was analyzed for sulfate with a Dionex model 2000i ion chromatograph equipped with a Dionex AS4A column and a conductivity detector. The eluent for sulfate analysis was 3.0 mM $NaHCO_3$ and 2.5 mM $NaCO_3$ at a flow rate of 2 ml min^{-1}. In preparation for heavy metal analysis, Pine and Hudson river sediments (1 g each, duplicate samples) were digested with concentrated HCl and concentrated HF (Spiers et al., 1983). Analysis of heavy metals (i.e. Pb, Cr, Cd, Cu, and Zn) was performed using an ARL D.C. Plasma Emission Spectrophotometer V-B.

Dechlorination assay. The ability of sediments collected from the Pine River and the Hudson River to support an anaerobic PCB-dechlorinating consortium was performed by using the transfer technique previously described (Quensen et al., 1990). In summary, anaerobic glass Balch tubes (28 ml) containing 1 g of sediment (sediment A from the Hudson River and sediments B, C, D, and E from the Pine River), a 2 ml suspension of microorganisms eluted from clean Hudson River sediments with revised anaerobic mineral medium (RAMM) (Shelton and Tiedje, 1984) and 2 μl/ml ethanol were incubated at 37°C until methane was detected in the headspace. Following this preincubation step, the tubes were autoclaved at 121°C for 1 h. Microorganisms (5 ml suspension) eluted from PCB-contaminated sediments were then added to the tubes. A 10% (wt/vol) solution of Aroclor 1242 in acetone (5 μl/g of sediment) was added to each tube for a final concentration of 0.5 mg/g air-dried sediment. Tubes were set up in triplicate, shaken overnight, and incubated at 25°C in the dark until time for sampling.

Debromination assay. Inocula were prepared from Pine River sediments collected from 4 sites downstream of the area of highest PBB contamination (Fig. 1). Samples of each sediment were placed in 2 l flasks which had been flushed with N_2:CO_2 (80:20). RAMM was added at approximately a 1:1 ratio of sediment to medium (w/v). The sediment slurry was shaken, and allowed to settle for approximately 10 min. Five ml of the supernatant were transferred to preincubated and autoclaved Balsch tubes (prepared as above) containing 1 g of air-dried non-PCB contaminated Hudson River sediment. The commercial PBB mixture, Firemaster BP-6 (obtained from Dr. M. Zabik, Dept. of Entomology, Michigan State University), dissolved in acetone (5 μl/g of sediment), was added at a rate of 0.5 mg/g dry sediment to each tube. The tubes, set up in triplicate, were shaken overnight, and incubated at 25^{o}C on a shaker in the dark until sampling.

Analyses. Just prior to sampling, methane was measured in the headspace of the Balsch tubes using a Carle Model AGC-111 gas chromatograph equiped with a 6 m Porapak Q column and microthermistor detector with Argon as the carrier gas (20 ml/min). PCBs and PBBs were sampled, extracted and analyzed on a capillary gas chromatograph with an electron capture detector according to Quensen et al. (1990). Polybrominated biphenyls were analyzed similarly, with the following modifications: detector 350^{o}C, and column at 160^{o}C for 1 minute, then 2^{o}C/min to 300^{o}C, and hold for 30 min. The PBB standard mixture was composed of congeners obtained from Ultra Scientific (Hope, R.I.), and from congeners purified from the Firemaster mixture (Dannan et al., 1982). Other PBB congeners were estimated by correlation with PCB retention times and response factors, and confirmed by mass spectrum data. Table 1 describes the congeners used in the PBB chromatographic standard.

TABLE 1. Polybrominated biphenyl (PBB) structure, number of bromines associated with each chromatographic peak, and average molecular weight.

Peak #	IUPAC #	Structure	Ortho	Total	Mol Wt
1	1	2-BB[a]	1	1	233.0
2	2	3-BB[a]	0	1	233.0
3	3	4-BB[a]	0	1	233.0
4	4	2-2-BB[a]	2	2	312.0
5	10	26-BB[a]	2	2	312.0
6	9	25-BB[a]	1	2	312.0
7	8	24-BB[a]	1	2	312.0
8	15	4-4-BB[a]	0	2	312.0
9	30	246-BB[a]	2	3	391.0
10	18	25-2-BB[a]	2	3	391.0
11	17	24-2-BB[b]	2	3	391.0
12	29	245-BB[a]	1	3	391.0
13	26 25	25-3-[a] or 24-3-BB[b]	1	3	391.0
14	31	25-4-BB[a]	1	3	391.0
15	28	24-4-BB[b]	1	3	391.0
16	53	26-25-BB[a]	2	4	470.0
17	51	24-26-BB[b]	3	4	470.0
18	38	345-BB[a]	0	3	391.0
19	52	25-25-BB[a]	2	4	470.0
20	49	24-25-BB[a]	2	4	470.0
21	47	24-24-BB[b]	2	4	470.0
22	103 80	246-25-BB[a]	3	5	549.0
23	101	245-25-BB[a]	2	5	549.0
24	110	236-34-BB	2	5	549.0
25	155	246-246-BB[a]	4	6	628.0
26	118	245-34-BB	1	5	549.0
27	133	235-235-BB[b]	2	6	628.0
28	146	235-245-BB[b]	2	6	628.0
29	153	245-245-BB[a]	2	6	628.0
30	141	2345-25-BB[b]	2	7	628.0
31	138	234-245-BB[c]	2	6	628.0
32	158 178	2346-34-[b] or 2356-235-BB[b]	2.5	6.5	667.5
33	167	245-345-BB[c]	1	6	628.0
34	156	2345-34-BB[c]	1	6	628.0
35	180	2345-245-BB[c]	2	7	707.0
36	194	2345-2345-BB[c]	2	8	786.0

[a] Obtained from Ultra Scientific (Hope, R.I.).

[b] The identity of these debromination products which were not present in the calibration mixture was inferred from their theoretical retention times (a linear relationship was derived for retention times of PCBs and PBBs in the standard mixture) and confirmed by mass spectroscopy.

[c] Obtained from Dr. S. Aust (from purification of congeners found in Firemaster).

Results and Discussion

Congener-specific analyses of the Pine River core samples were performed to evaluate whether *in situ* reductive debromination of the Firemaster mixture had occurred. Evidence for *in situ* debromination would be detection of non-Firemaster congeners, or of relative increases in lesser brominated biphenyls present in Firemaster, that could result from the debromination of congeners present in Firemaster. Total PBB concentrations and the proportion of non-Firemaster peaks present in each core sample are shown in Figure 2. Core 2 contained the highest concentration of total PBBs, 125 μg /g, at a sediment depth of 25 cm. Core 3, which was located downstream of the predominant site of contamination, reached a maximum total PBB concentration of approximately 2.3 μg/g at a depth of 15 cm. All three cores contained non-Firemaster PBB congeners, including 2-2'-BB, 2,4-BB, 2,5-2'-BB, 2,4-2'-BB, 2,5-3'-BB, (co-elutes with 2,4-3'-BB), 2,4-4'-BB, 2,5-2',4'-BB, and 2,4-2',4'-BB (data not shown). All of these PBB congeners are possible debromination products of Firemaster produced by the selective removal of *meta* and *para* bromines. Thus the congener profiles obtained for the Pine River sediments suggest that *in situ* debromination of Firemaster has occurred.

The extent of *in situ* debromination, however, is rather limited. Cores 1 and 2 contained 10 -12% non-Firemaster PBBs. PCB *in situ* dechlorination has been shown to account for dechlorination of 74% (Quensen et al., 1991) and 83% (Brown et al., 1988) of the *meta* plus *para* chlorines found in the Upper Hudson River. The higher proportion of non-Firemaster PBBs (50-65%) depicted for Core 3 (Figure 2) may be an artifact. Because the total PBB concentrations for these core sections were so low (0.6 to 2.4 ppm), concentrations for the smaller peaks detected were below the limits for reliable quantitation.

The above results are only suggestive of *in situ* reductive debromination of PBBs, in part because the composition of the Firemaster originally input to these

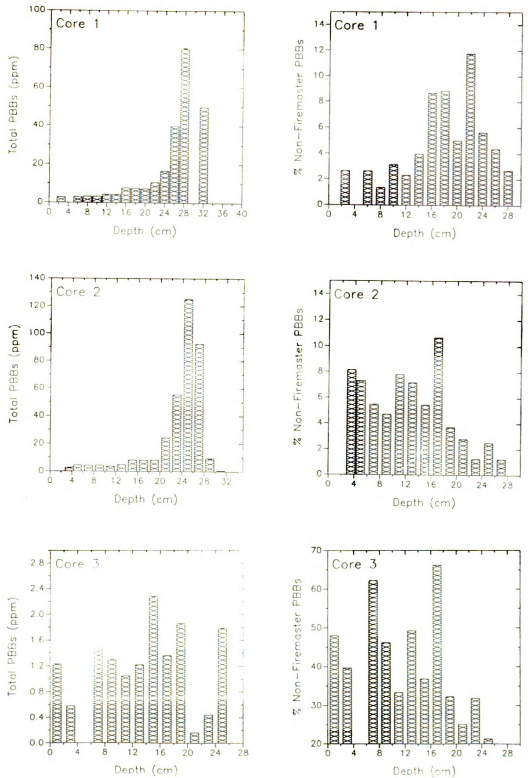

FIGURE 2. Total PBB concentration and the percentage of the total PBBs represented by PBB congeners which are not found in the PBB mixture (Firemaster) over depth. Cores 1, 2, and 3 (refer to Figure 1 for sampling locations) were analyzed by for PBBs by congener-specific analysis.

sediments is unknown and probably varied over time. For example, the proportion of 2,4,5-2',4',5'-BB ranged from 50 to 70% of the total mixture (Fries, 1984). It is probable that lesser brominated Firemaster mixtures contained some of the congeners we have classified as non-Firemaster PBBs based on our standard. The variation in the proportion of non-Firemaster PBBs with depth (Figure 2) may also be due in part to temporal variations in the degree of bromination of the PBBs input to the sediments.

To further address the possibility of *in situ* PBB debromination in the Pine River sediments, we assayed sediments from sites F - I for the presence of microorganisms capable of PBB debromination or PCB dechlorination. Dechlorination and debromination assays were conducted using microorganisms eluted from the four Pine River sediments which are known to support PCB dechlorination by microorganisms eluted from PCB-contaminated Hudson River sediments (Quensen et al., 1988; Quensen et al., 1990). None of the Pine River sediments tested contained microorganisms capable of dechlorination of the commercial PCB mixture Aroclor 1242 during 32 weeks of incubation. Sediments F, G, and I did not contain microorganisms capable of debrominating Firemaster. However, anaerobic microorganisms eluted from sediments collected from site H did debrominate the PBB mixture. Evidence for debromination by inoculum prepared from site I sediments is shown in Figure 3, where peaks 10 (2,5-2'-BB), 19 (2,5-2',5'-BB), 20 (2,4-2',5'-BB), and 21 (2,4-2',4'-BB) correspond to the major products of PBB debromination. The homolog distribution of PBB congeners at week 32 (Table 2) shows an increase in tri- and tetra-bromobiphenyls not present in commercial Firemaster with a concomitant decrease in the levels of penta-, and hexa-, and hepta-bromobiphenyls.

The demonstration that microorganisms capable of debrominating PBBs exist in the Pine River reservoir, and the fact that they produce several of the non-

TABLE 2. Distribution of PBB congeners of Firemaster after 32 weeks of incubation with microorganisms eluted from the Core 3 sediment of the Pine River.

Homolog Distribution	Mole % Week 32	Mole % Week 32 Control
Mono-	0	0
Di-	0	0
Tri-	2.50	0
Tetra-	5.71	0
Penta-	7.06	8.05
Hexa-	74.18	80.55
Hepta-	10.55	11.41
Octa-	0	0

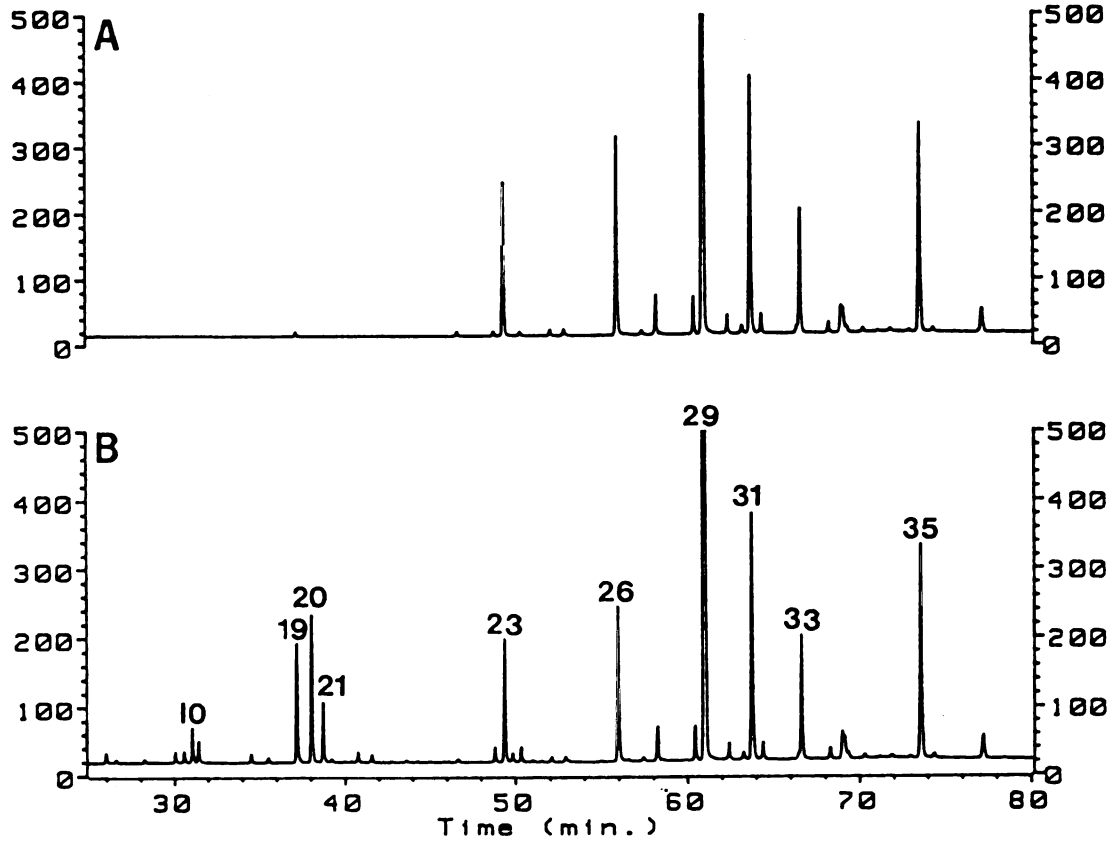

FIGURE 3. Gas chromatogram of (A) Firemaster after 32 weeks of incubation with autoclaved Pine River microorganisms from site H and non-PCB contaminated Hudson River sediment, and (B) Firemaster after 32 weeks of incubation with Pine River microorganisms from site H and non-PCB contaminated Hudson River sediment. Peak assignments are given in this publication, Chapter 2, Table 1.

Firemaster peaks observed in sediments of the Pine River reservoir strengthens our argument that limited *in situ* debromination has occurred. The reservoir sediments are known to be heavily contaminated with petroleum wastes from an upstream oil refinery, heavy metals, and halogenated compounds other than PBBs. To investigate how these co-contaminants might influence the anaerobic dehalogenation process, we collected sediments from the most heavily contaminated site (E) in the reservoir and from sites progressively upstream and presumably less contaminated. We than assayed these sediments for their ability to support the reductive dechlorination of Aroclor 1242 by anaerobic microorganisms eluted from PCB-contaminated Hudson River sediments. These microorganisms have demonstrated capability to reductively dechlorinate PCBs in the presence of non-contaminated Hudson River sediments (Quensen et al., 1988; Quensen et al., 1990).

When the abilities of the various Pine River sediments to support dechlorination were compared to the ability of clean Hudson River sediment, it was evident that the Hudson River sediment supported the most dechlorination during a 32 week incubation (Figure 4). Sediment E, from the site in the Pine River that is most heavily co-contaminated, did not support microbial CO_2 or CH_4 production, or dechlorination during the 32 weeks of incubation (data not shown). Pine River sediments B,C, and D all supported PCB dechlorination to some extent. Of the sediments that supported dechlorination, sediment D had the longest acclimation period (12 weeks). Dechlorination in the presence of the other sediments occurred by week 4. Pine River sediment C supported the most dechlorination by week 4 but after week 8 dechlorination activity leveled off in sediment C while continuing in sediment A.

In order to understand more specific sediment factors that may be inhibiting dehalogenation activity (e.g., absence of microorganisms with dehalogenation activity, sediment toxicity), the sediments previously tested for their ability to

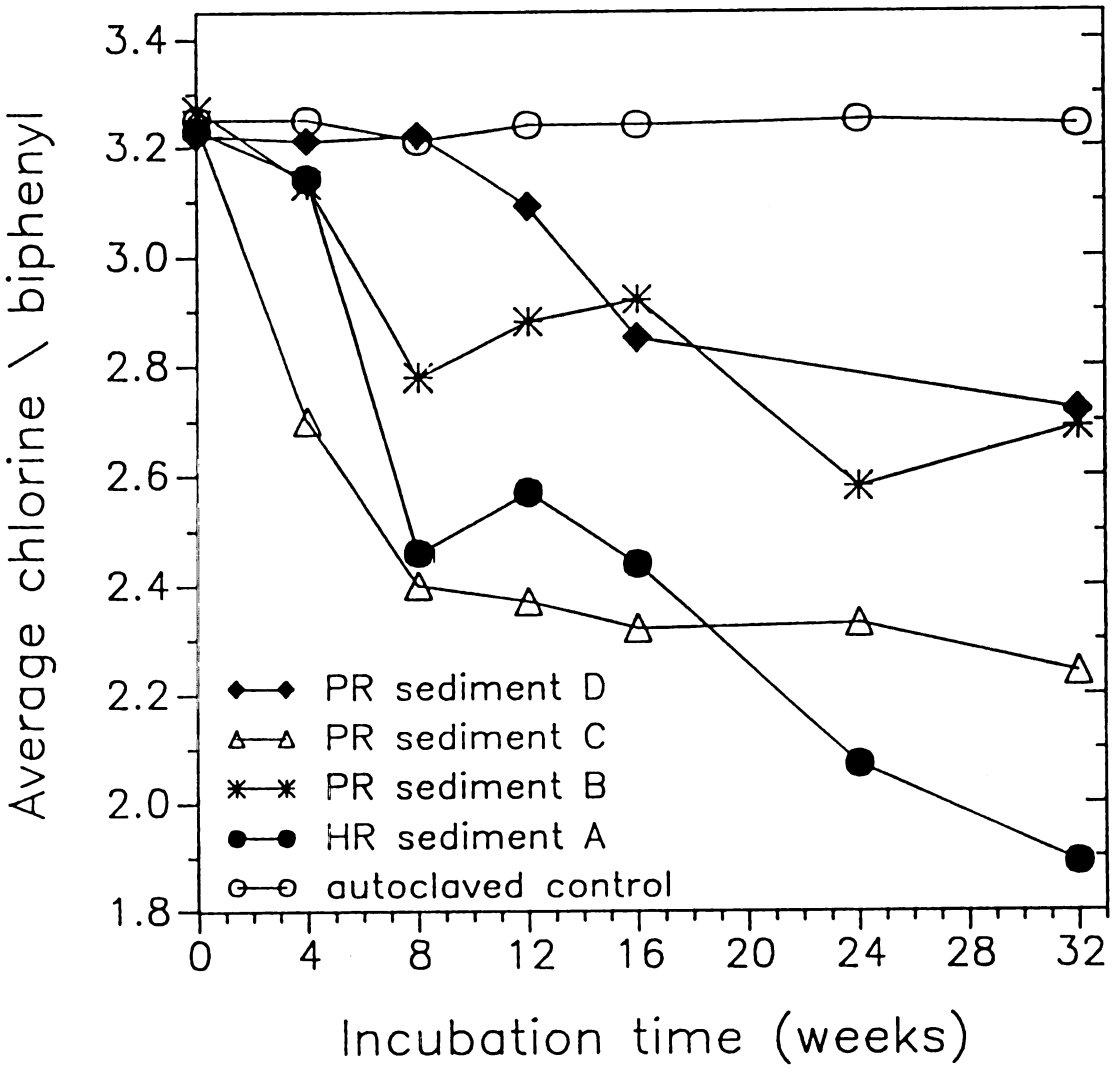

FIGURE 4. Reductive dechlorination of Aroclor 1242 by anaerobic microorganisms eluted from Hudson River sediment in the presence of non-PCB contaminated Hudson River sediment (A) and four Pine River sediments (B,C, and D, refer to Figure 1 for sediment sampling locations). Pine River sediment E was also used, but did not support reductive dechlorination of Aroclor 1242 in the 32 week incubation.

support dechlorination were characterized. Organic carbon, sulfate, oil and grease, heavy metals, and particle size distribution contents of Pine and Hudson River sediments are shown in Table 3. Sediment A is from a non-PCB contaminated region of the Hudson River. Sediments B, C, and D were collected from the Pine River at locations upstream from the PBB manufacturing site and sediment E is from a heavily contaminated area of the Pine River (refer to Figure 1 for sampling locations) adjacent to the PBB manufacturing site. Sediments A, B, and C were predominately sandy, whereas sediments D and E had higher contents of silt and clay-sized particles. These latter sediments were obviously from more depositional sites in the Pine River Reservoir. Sediment B was exceptionally low in organic carbon. Sediment E contained the highest concentrations of sulfate, oil and grease, and heavy metals. Sediments A, B, and C all contained less than 0.1% oil and grease, and considerably lower levels of heavy metals; sediment D had levels of these co-contaminants intermediate between sediment E and sediments A, B, and C.

Cores 1 and 2 were sampled from sites in the Pine River containing the highest concentrations of organic co-contaminants (Forba, 1980; Forba, 1982). Core 1 was in close proximity to sediment E which contained the highest oil and grease content and heavy metal loadings. Each of these factors could reasonably inhibit dehalogenation. This general location in the Pine River is also contaminated with 13 known halogenated organic compounds and numerous hydrocarbons (Forba, 1980; Forba, 1982), as well as compounds detected by mass spectroscopy that contained both chlorine and bromine substituents (data not shown). The predominate halogenated compounds detected were PBBs, p,p'-DDT, DDD, DDE, hexabromobenzene, chlorobenzene, dichlorobenzene and chlordane (Forba, 1980; Forba, 1982). Sediment from this site (sediment E) did not support reductive dechlorination of Aroclor 1242 by microorganisms from the Hudson River that are

TABLE 3. Characterization of Hudson River (A) and Pine River (B,C,D and E) sediments.

	Sediment				
	A	B	C	D	E
O.C.(%)	7.66	0.09	1.83	4.63	5.25
SO_4^{2-} (μg/g)	449	175	409	320	4775
oil & grease (%)	0.28	0.09	0.08	0.56	1.93
% sand	88.9	94.3	69.1	21.8	39.8
% silt	7.4	2.0	14.7	56.0	46.0
% clay	3.7	3.7	16.2	22.2	14.2
heavy metals					
Pb (μg/g)	257	74	106	467	630
Cr (μg/g)	63	29	33	336	247
Cd (μg/g)	5.25	1.15	1.85	6.83	4.30
Cu (μg/g)	36	9.4	11.05	161	972.5
Zn (μg/g)	169.2	47.5	40.6	444.2	245.0
Supports Dechlorination	+++	+	++	+	-

capable of dechlorinating Aroclor 1242 in the presence of non-PCB contaminated Hudson River sediments. The sediment E site in the Pine River contained 2% oil and grease by weight. It has been observed that residual oil components of soils, like natural organic matter, can act as a partition media for organic compounds (Sun and Boyd, 1989). The high oil and grease content in Pine River sediments at this site could act to sequester the PBBs, and hence strongly influence their bioavailability to microorganisms. It has been previously observed under aerobic conditions with PCBs that the addition of humic acid inhibited both the rate and extent of PCB degradative activity, and in similar studies, the addition of mineral oil even more severely inhibited the biodegradation of PCBs (Harkness and Bergeron, 1990). Sun and Boyd (1991) suggest a threshold residual oil concentration of about 0.1% (w/w) above which a separate sorptive phase may form. These phases function as highly effective partitioning medium and could substantially reduce the availability of organic contaminants to biodegradative processes.

Additionally, sediment eluent from (F) located near core 1 could not dehalogenate PCBs or PBBs in the presence of non-PCB contaminated Hudson River sediments. Sediment from this region of the Pine River (site E) had extremely high concentrations of sulfate. The presence of high concentrations of sulfate at this location suggests that there was little biological activity, since sulfate is a favorable electron acceptor and would be consumed in an anaerobic environment.

One site in the Pine River Reservoir, located downstream from the site of the heaviest contamination, contained microorganisms which could reductively debrominate Firemaster, but not dechlorinate Aroclor 1242 (site H). It has been previously observed that methanogenic freshwater lake sediment acclimated to iodobenzoate dehalogenation did not cross-acclimate to chloro- or bromobenzoates, suggesting two different types of dehalogenating activity (Horowitz et al., 1983). Likewise, microorganisms found to reductively dechlorinate Aroclor 1260 (which

averages 6 chlorines per biphenyl molecule) were unable to debrominate Firemaster (which averages 6 bromines per biphenyl molecule) (Morris, this publication, Chapter 3). These Pine River sediment microorganisms removed bromines from the *meta* and *para* position of Firemaster, as observed previously with PCBs (Quensen et al., 1988; Quensen et al., 1990), while *ortho*-substituted bromines were not removed. This site, which was contaminated with only several ppm of total PBBs, had approximately half of the total PBBs as non-Firemaster PBBs, strongly indicating in situ debromination. Several non-Firemaster PBB congeners from in these sediments were also observed in laboratory PBB incubations utilizing organisms eluted from this site and non-contaminated Hudson River sediments. Previous examination of this part of the Pine River Reservoir (Forba, 1980; Forba, 1982) showed that it was less co-contaminated with halogenated organics (e.g., DDT and PBBs) than the Core 1 and 2 sampling sites.

Summary

While reductive dechlorination of PCBs in sediments has been observed in several locations (Brown et al., 1984; Brown et al., 1987a; Brown et al., 1987b; Brown and Wagner, 1990), it is a process that requires not only microorganisms capable of the dechlorination reaction, but also the proper environment. In this study, there was not strong evidence for extensive debromination of the commercial PBB mixture, Firemaster, in heavily contaminated sediments of the Pine River located in close proximity to the PBB manufacturing site. However, *in situ* debromination was indicated in Pine River sediments at one site downstream of the heaviest contamination. Organisms eluted from these sediments were capable of debrominating PBBs in the presence of clean Hudson River sediments. Sediments from the Pine River upstream of the site of heaviest contamination were able to support reductive dehalogenation of Aroclor 1242 by Hudson River

microorganisms, however the most contaminated region of the Pine River was not able to support dehalogenation activity. These results suggest that co-contaminants, such as petroleum products and heavy metals, could inhibit *in situ* dehalogenation.

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Chapter 3:

Reductive Debromination of the Commercial Polybrominated Biphenyl Mixture, Firemaster FM-BP6, by Anaerobic Microorganisms from Sediments

Introduction

Polybrominated biphenyls (PBBs) are a group of industrial compounds that were manufactured as fire retardants for thermoplastic applications. The commercial production of PBBs began in 1970, and approximately 13.3 million pounds were produced in the U.S. between 1970 and 1976 (Di Carlo et al., 1978). The Michigan Chemical Corporation (later Velsicol Chemical Corporation, St. Louis, Michigan) manufactured PBBs under the trade name Firemaster BP-6 between 1970 and 1974. In 1973 between 500 to 1,000 pounds of the flame retardant was mistaken for the dairy feed additive magnesium oxide, resulting in contamination of animal feeds, animals, and soils in Michigan (Carter, 1976). The Michigan Chemical Corp. manufacturing site (Velsicol) is located on the Pine River in St. Louis, Michigan. Sediments at this site are heavily contaminated with PBBs, as well as with a number of other halogenated compounds including DDT, hexabromobenzene, chlordane), petroleum products and heavy metals (Forba, 1980).

Commercial PBBs were produced by bromination of biphenyl with elemental bromine in the presence of a catalyst; this process yielded a product less complex than commercial polychlorobiphenyl mixtures (Aroclors). Greater than 50% of the PBB mixture is comprised of a single congener, 2,4,5-2',4',5'-hexabromobiphenyl (Sundstrom et al., 1976), whereas commerical Aroclors commonly consist of up to 90 PCB congeners. Polybrominated biphenyls, like PCBs, were considered to be highly recalcitrant in the environment, and there is evidence for their environmental persistence (Jansson and Asplund, 1987). Their behavior in soils, which is characterized by low leachability (Filonow et al., 1976), lack of plant uptake (Jacobs et al., 1976; Chou et al., 1978), and absence of biological degradation (Jacobs et al., 1976; 1978) contributes to their environmental persistence.

Recently, Brown and colleagues (1987a; 1987b) provided evidence for *in situ* reductive dechlorination of PCBs in anaerobic sediments from the Hudson River. Quensen et al. (1988; 1990) subsequently demonstrated that anaerobic microorganisms eluted from PCB-contaminated sediments could dechlorinate PCBs (as Aroclor mixtures) which were added to previously uncontaminated sediments. This anaerobic reductive dechlorination of PCBs renders the Aroclor mixtures less toxic, and more susceptible to aerobic degradation processes.

Although reductive dehalogenation has been shown to occur with PCBs, no studies have been conducted with PBBs. In general, bromine is lost from haloaliphatic compounds more readily than chlorines (Vogel et al., 1987). In this report, we compare the reductive debromination of PBBs by anaerobic microorganisms from three sites; a Firemaster-contaminated (PBB) sediment (Pine River, MI), an Aroclor 1242-contaminated (PCB) sediment (Hudson River, NY), and an Aroclor 1260-contaminated (PCB) sediment (Silver Lake, MA).

Materials and Methods

Sediments. Aroclor 1242-contaminated sediments were collected in August 1988 from the Hudson River at River Mile 193.5 near Hudson Falls, NY (site H7 in Brown et al., 1987). Clean (non-PCB-contaminated) Hudson River sediments were collected upstream at River Mile 205. Aroclor 1260-contaminated sediments were collected from Silver Lake near Pittsfield, MA in September 1989 (Brown et al., 1987a; Brown et al., 1987b). The Silver Lake sediments additionally contained high concentrations of oil and polyaromatic hydrocarbons from a coal gasification plant. Polybrominated biphenyl (Firemaster)-contaminated sediments were collected from the Pine River reservoir (St. Louis, MI) adjacent to the Michigan Chemical Corp. site just upstream from the hydroelectric dam (refer to Table 1, Chapter 2, this

publication). In addition to PBBs, these sediments contained a variety of co-contaminants, including hexabromobenzene, DDT, heavy metals, and petroleum products (Forba, 1982; Morris, this publication, Chapter 2). Aroclor 1242- and 1260-contaminated sediments were collected with a post hole digger to a depth of approximately 25 cm and transported to the laboratory in tightly sealed paint cans. Firemaster-contaminated sediments were collected by pushing 3 inch PVC pipes into the sediments to approximately 25 cm to retrieve the sediment, and then capped for transportation to the laboratory.

Preparation of anaerobic inoculum. Contaminated and non-contaminated sediments (described above) were placed in 2 liter Erlenmeyer flasks that were flushed with O_2-free N_2/CO_2 (80:20, vol/vol) using a Hungate apparatus. Equal volumes of sediment and reduced anaerobic mineral medium (Shelton and Tiedje, 1984) were placed in the flasks and sealed with butyl rubber stoppers. The flasks were shaken by hand for approximately 2 min. and allowed to settle for approximately 10 min. The supernatants were subsequently withdrawn under a N_2/CO_2 atmosphere and were then used as inocula for the dehalogenation experiments.

Assay Vessels. Two dehalogenation assays were used in this study. For experiments conducted with Hudson River, Pine River, and Silver Lake microorganisms which were incubated with mineral medium alone (described below), glass Balch tubes (28 ml) were used. For experiments utilizing amended mineral medium (described below) and microorganisms from a pyruvate and Aroclor 1242 enrichment culture (Morris, Chapter 4, this publication) organisms were incubated in glass serum bottles (160 ml).

Medium. The reduced anaerobic mineral medium (RAMM) of Shelton and Tiedje (1984) was used for all experiments set up in glass Balch tubes (28ml). For

experiments set up in serum bottles, this medium was amended as follows: 1 mM cysteine instead of Na_2S, 10 mM instead of 4 mM potassium phosphate buffer (pH 7.0), $NaHCO_3$ omitted, vitamins and 0.1 mM titanium (III) citrate added. Vitamins, titanium (III) citrate and sodium pyruvate (20 mM) were added after autoclaving from filter-sterilized stock solutions. Vitamins included a mixture of vitamins generally used by anaerobes (Wolin et al., 1963) plus 450 μg/l nicotinamide and 200 μg/l naphthoquinone (DeWeerd et al., 1990). Titanium (III) citrate was prepared according to Zehnder and Wuhrmann (1976). The pH was adjusted to 7.0 before autoclaving.

Cultures. Clean Hudson River sediment was air-dried and sieved through a 2 mm mesh screen. For experiments using Balch tubes, 1 g of these sediments was weighed into each glass Balch tubes, and flushed with O_2-free N_2 using a Hungate apparatus. The sediments were preincubated to ensure strict anaerobic conditions by adding 2 ml of inoculum eluted from clean Hudson River sediments and ethanol (1 μl/ml) to each tube. Tubes were incubated at 37^{o}C in the dark until methane was detected in the headspace (analysis described below). After methane production was observed, indicating anaerobic conditions, the tubes were autoclaved for 1 h at 121^{o}C. After autoclaving, 5 mls of inoculum from PCB-contaminated or PBB-contaminated sediments were added to each tube. All experiments in tubes were set up in triplicate.

For experiments with the pyruvate plus Aroclor 1242 enrichments using glass serum bottles (160 ml), 25 g of sieved, air-dried clean Hudson River sediments were added to each bottle, along with 40 ml of amended RAMM and 10 ml of inoculum. In these experiments, the preincubation step (for methane production) was eliminated. All experiments utilizing serum bottles were set up in duplicate.

Firemaster and Aroclor addition. A 10% (wt/vol) solution of Aroclor (1242 or 1260) (Monsanto Co., St. Louis, Mo.) and Firemaster (obtained from Dr. M. Zabik, Dept. of Entomology, Michigan State University) in acetone was added to each tube (or serum bottle) while flushing with O_2-free N_2. to give a final concentration of 500 μg or 50 μg of PCBs or PBB per g sediment. The PBB congener, 2,4,5-2',4',5'-BB (Ultra Scientific, Hope, R.I.), was added to separate tubes at 250 μg or 25 μg per g of sediment. After PCB or PBB addition, the tubes were resealed with teflon-coated rubber stoppers (West Co., Phoenixville, Pa.) and incubated at 25^{o}C in the dark.

Analyses. Tubes and bottles were sampled and extracted according to Quensen et al., 1990. Headspace gas analysis (primarily for CH_4 detection) was determined with a Carle Model AGC-111 gas chromatograph with a 6 m Porapak Q column and microthermistor detector with argon as the carrier gas. Congener-specific analysis was performed using a gas chromatograph and an electron capture detector as described previously for PCBs by Quensen et al. (1990). Conditions for PBB identification were the same as for PCBs with the following modifications: detector 350^{o}C, and column at 160^{o}C for 1 minute, then 2^{o}C/min to 300^{o}C, and hold for 30 min. The PBB standard mixture was composed of congeners obtained from Ultra Scientific (Hope, R.I.), from congeners purified from the Firemaster mixture (obtained from Dr. S. Aust, Dannan et al., 1982), and those estimated by correlation with PCB retention times, response factors and mass spectrum data. The congeners used in the PBB standard, as well as the values used for molecular weight, *ortho* and total bromine per biphenyl, and peak number assignment are given in Chapter 2, Table 1.

Results

Dehalogenation of Firemaster and Aroclor 1242 by Hudson River microorganisms. We compared the dechlorination of Aroclor 1242 to the debromination of Firemaster (PBB) by Hudson River microorganisms previously shown to be capable of PCB dechlorination (Quensen et al., 1988). Dechlorination of Aroclor 1242 (at 500 μg/g) was observed at week 8 (Figure 1A), and was more rapid than debromination of Firemaster (Figure 1B). In this experiment, Hudson River microorganisms removed 29% of the *meta* and *para* bromines from Firemaster, compared to 59% of the *meta* and *para* chlorines (for an average of one chlorine removed per biphenyl molecule) from Aroclor 1242 during a 40 week incubation. No *ortho* chlorine removal was observed.

Dehalogenation of Firemaster and Aroclor 1260 by Silver Lake microorganisms. The dehalogenation of Firemaster and Aroclor 1260 (both at 500 μg/g) by Silver Lake microorganisms was compared. These organisms were eluted from sediments contaminated with Aroclor 1260. Aroclor 1260 and Firemaster both average roughly 6 halogens per biphenyl molecule. Chlorines from the *meta* and *para* positions of Aroclor 1260 decreased 18% (Figure 2A), and no *ortho* dehalogenation was observed. There was a 24 week lag in dechlorination activity for Aroclor 1260. There was no evidence of debromination of Firemaster by Silver Lake microorganisms (Figure 2B).

Reductive debromination of Firemaster by Pine River, Hudson River, and Silver Lake microorganisms. The abilities of microorganisms eluted from PCB-contaminated sites (Hudson River, Silver Lake) and a PBB-contaminated site (Pine River) to debrominate Firemaster at 50 and 500 μg/g sediment were compared directly. Debromination was observed at the higher Firemaster concentration (Figure 3), but no significant debromination by microorganisms from the three sites

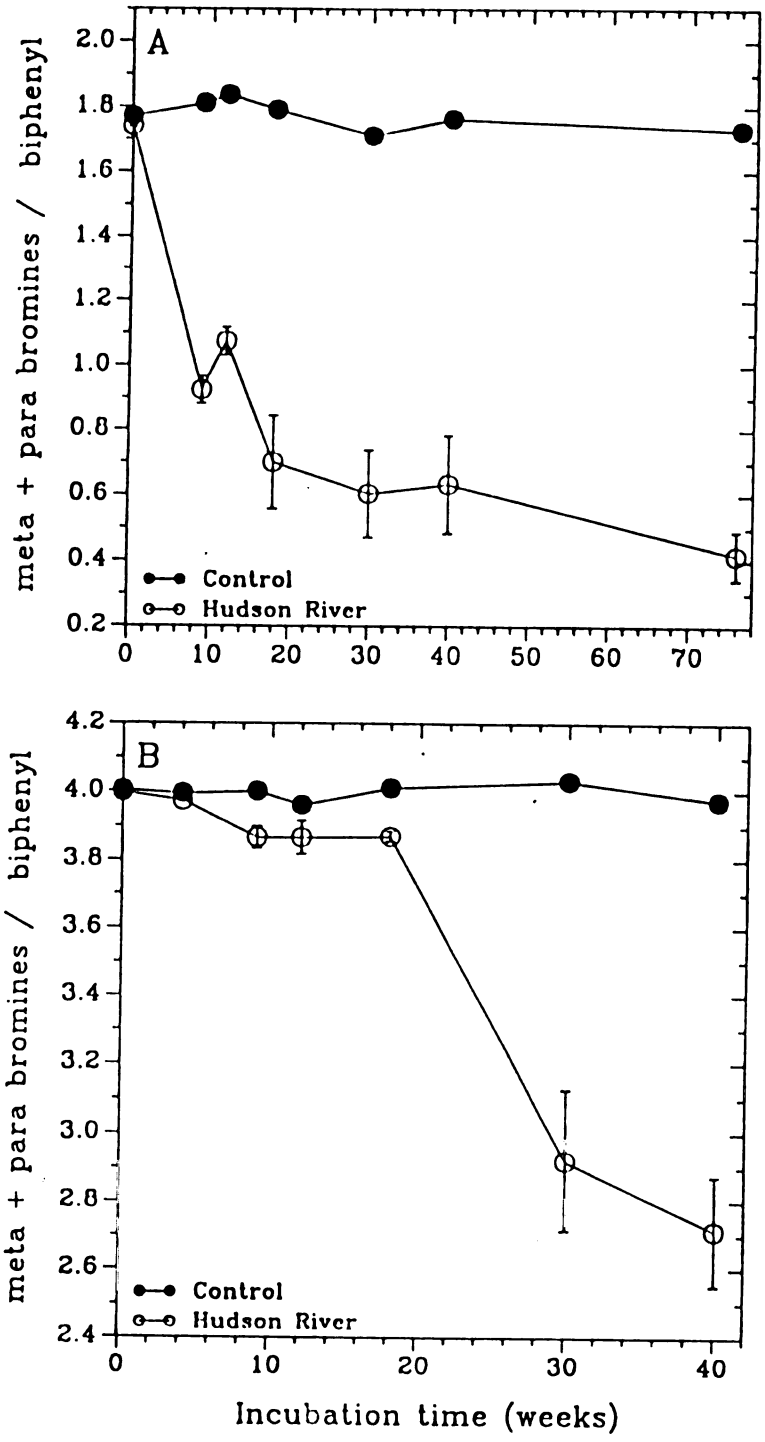

FIGURE 1. Reductive dehalogenation of PCBs and PBBs by Hudson River microorganisms. (A) Dechlorination of Aroclor 1242, (B) Debromination of Firemaster. Data are means of triplicates.

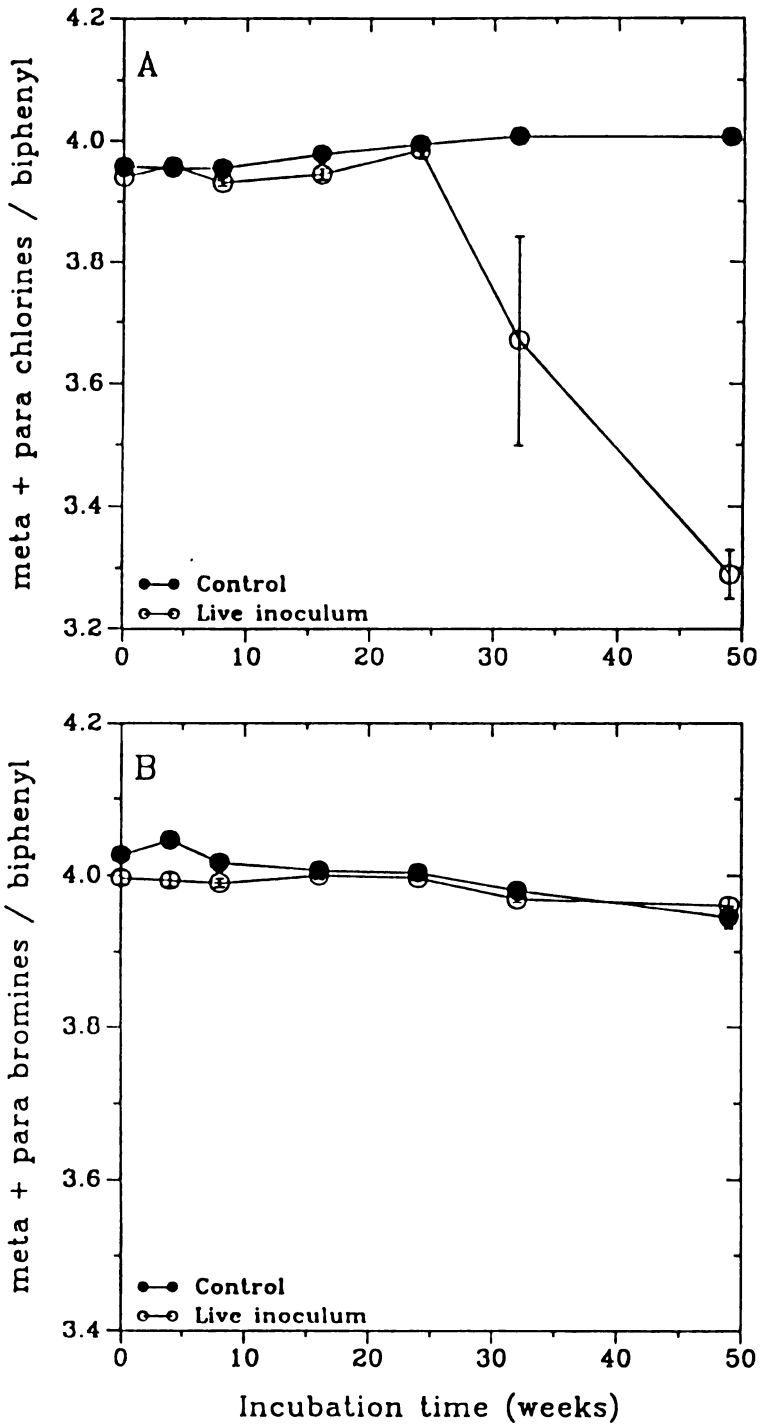

FIGURE 2. Reductive dehalogenation of (A) PCBs (Aroclor 1260) and (B) PBBs (Firemaster) by Silver Lake microorganisms. Closed circles, autoclaved; open circles, live inoculum.

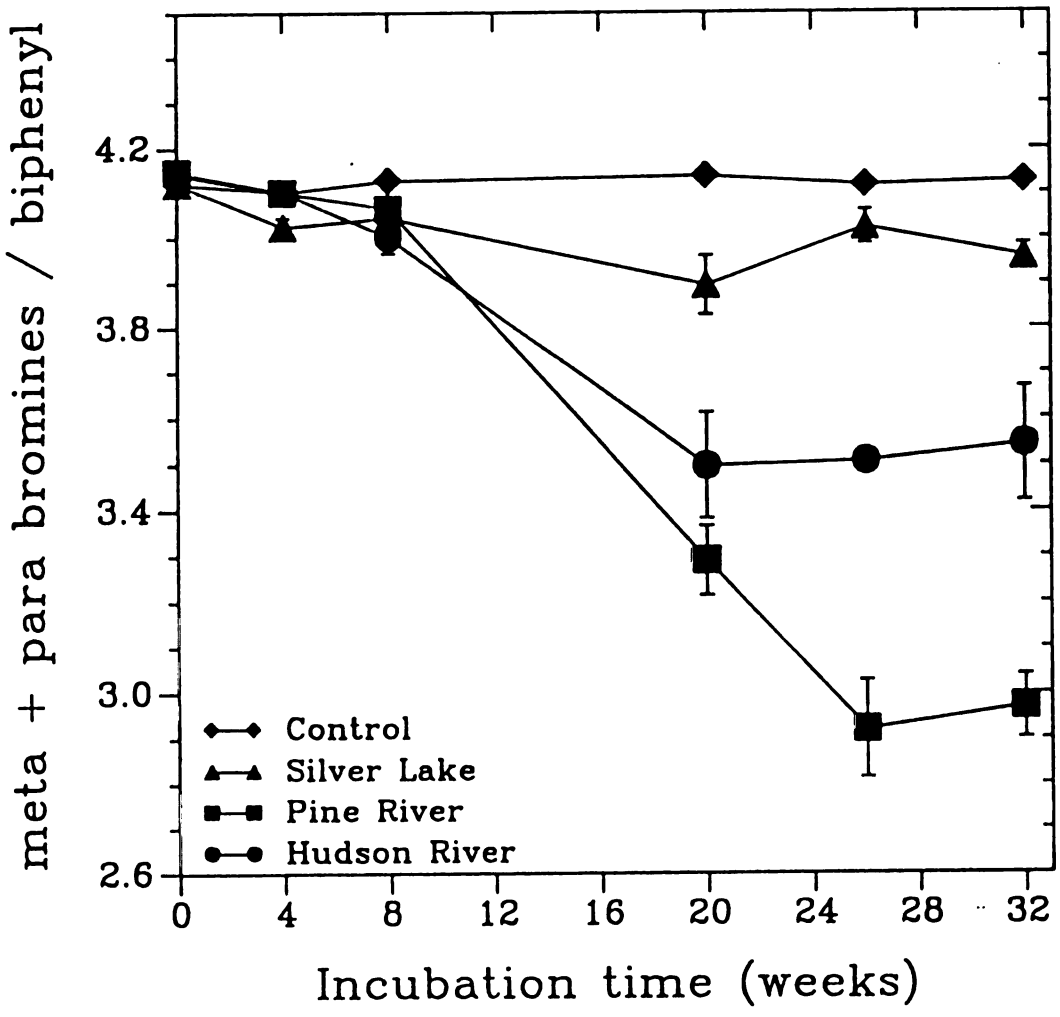

FIGURE 3. Reductive debromination of *meta* plus *para* bromines of Firemaster by Silver Lake, Pine River, and Hudson River microorganisms. Data are means of triplicates.

tested was observed at 50 μg Firemaster /g sediment. In this second attempt with Silver Lake microorganisms, 3% of the average number of *meta* plus *para* bromines per biphenyl were removed in the first 20 weeks of incubation (Figure 3). Debromination activity by the Hudson River microorganisms resulted in removal of 12% of the *meta* plus *para* chlorines. Organisms from the PBB-contaminated Pine River sediments showed the highest debromination activity among the three inocula, resulting in removal of 32% of the *meta* plus *para* chlorine. The Pine River microorganisms removed an average of one bromine per biphenyl molecule from Firemaster between 8 and 20 weeks of incubation, and a total of 1.25 bromines were removed during the 32 week experiment.

The appearance of products of Firemaster debromination by Pine River microorganisms, after 8 weeks of incubation, included 2,4-2',4'-BB (peak 21), 2,4-2',5'-BB (peak 20), 2,5-2',5'-BB (peak 19), 2,4-2'-BB (peak 11), 2,5-2'-BB (peak 10) and a small amount of 2-2'-BB (peak 4) (Figure 4). The major debromination products observed in incubations of Firemaster with the Hudson River, Silver Lake, and Pine River microorganisms included 2,5-2'-BB (peak 10) and to a lesser extent, 2,4,-2'-BB (peak 11) (Figure 5). While Silver Lake microorganisms showed limited debromination of Firemaster, several debromination products were observed, including 2,4-2'-BB (peak 11) and 2-2'-BB (peak 4) Figure 6). The extent of dehalogenation of Firemaster, Aroclor 1242, and Aroclor 1260 by microorganisms from the three study sites is summarized in Table 1.

Reductive debromination of 2,4,5-2',4',5'-bromobiphenyl. One PBB congener, 2,4,5-2',4',5'-BB (peak 29) represents roughly 53% (on a molar basis) of Firemaster, and this congener decreased 58% percent after 32 weeks of incubation with Pine River microorganisms (Figure 4). This congener decreased 33% and 16% after 32 weeks of incubation with Hudson River and Silver Lake microorganisms,

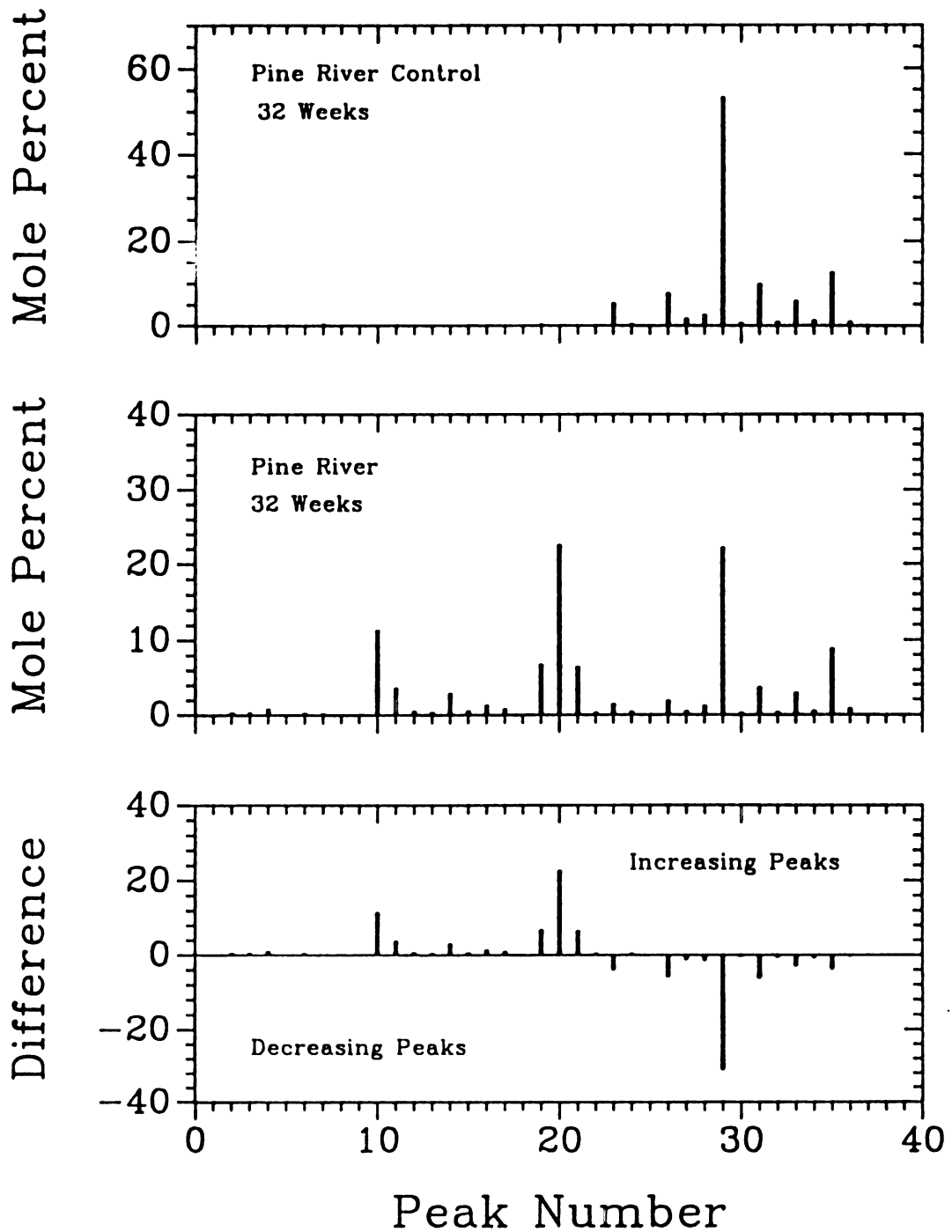

FIGURE 4. Mole percentage of PBBs represented by each chromatographic peak after 32 weeks of incubation of Firemaster with microorganisms eluted from Pine River sediments. The lower histogram shows the mole percent increase and decrease in PBBs.

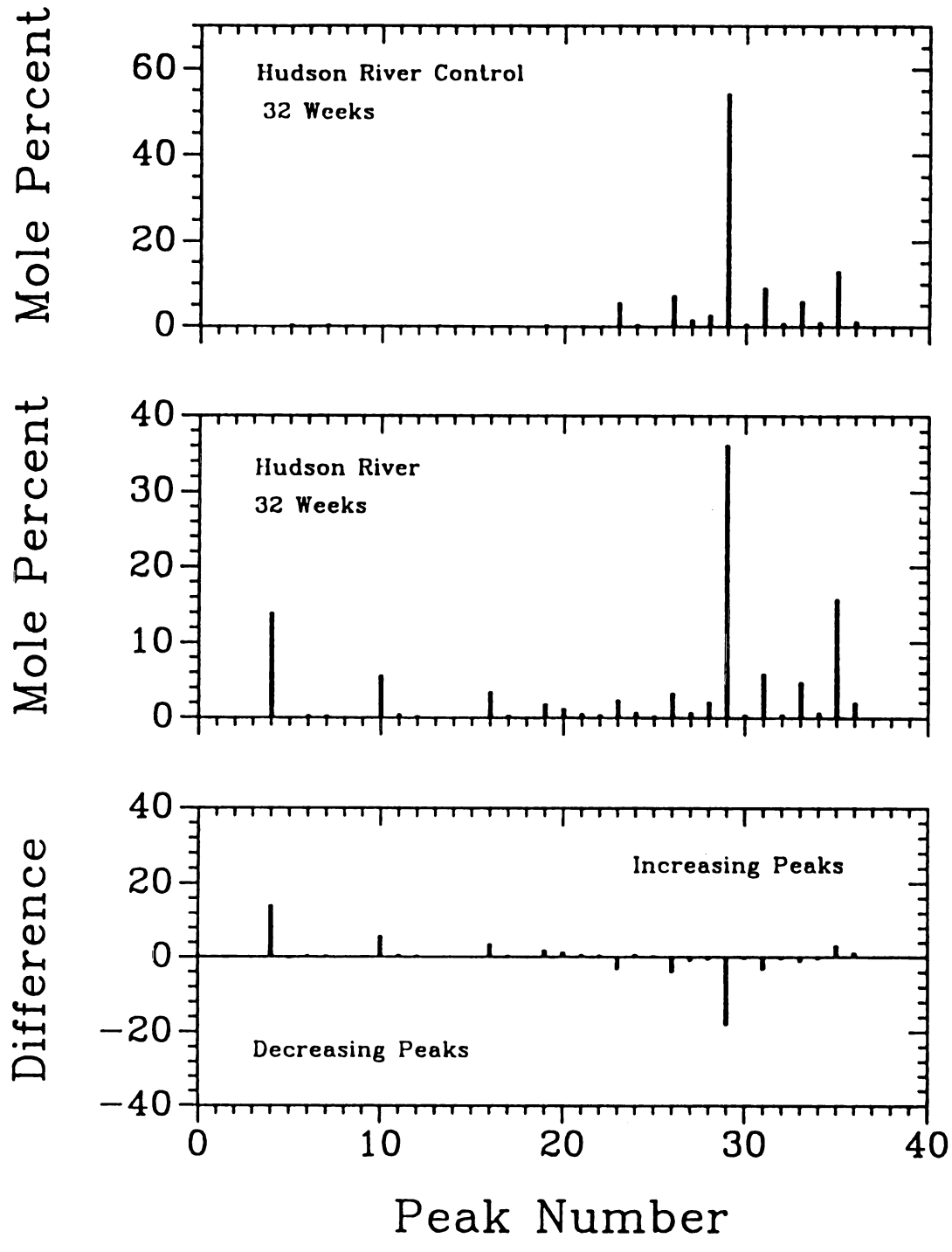

FIGURE 5. Mole percentage of PBBs represented by each chromatographic peak after 32 weeks of incubation of Firemaster with microorganisms eluted from Hudson River sediments. The lower histogram shows the mole percent increase and decrease in PBBs.

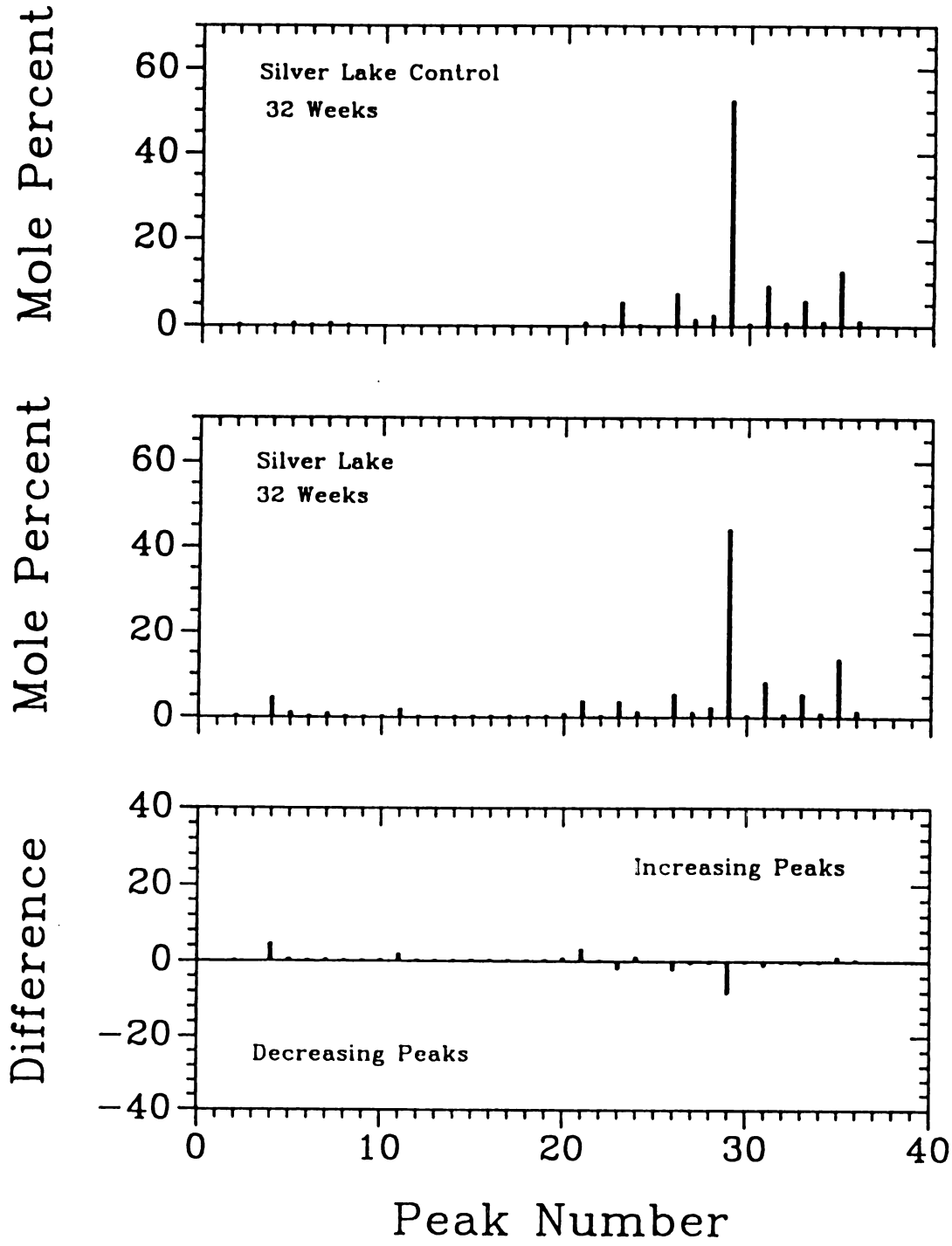

FIGURE 6. Mole percentage of PBBs represented by each chromatographic peak after 32 weeks of incubation of Firemaster with microorganisms eluted from Silver Lake sediments. The lower histogram shows the mole percent increase and decrease in PBBs.

TABLE 1. Extent of dehalogenation of Firemaster (PBBs) or Aroclors (PCBs) by microorganisms collected from a PBB contaminated site (Pine River), an Aroclor 1242 contaminated site (Hudson River), and an Aroclor 1260 contaminated site (Silver Lake).

Inoculum Source	Firemaster or Aroclor	Period	% *Meta* plus *Para* Halogen Removed
Hudson River	1242	40	59
	Firemaster	40	29
Silver Lake	1260	50	18
	Firemaster	50	0
Pine River	Firemaster	32	32
Hudson River	Firemaster	32	12
Silver Lake	Firemaster	32	3

respectively (Figure 5 and 6). Other congeners that were debrominated by the Pine River microorganisms included 2,4,5-3',4'-BB (peak 26), 2,3,4-2',4',5'-BB (peak 31), and 2,4,5-3',4',5'-BB (peak 33). Most of the debromination products could be accounted for by the debromination of 2,4,5-2',4',5'-BB. When 2,4,5-2',4',5'-hexabromobiphenyl (at 250 or 25 μg/g sediment) was incubated alone with Hudson River microorganisms, no measurable debromination was observed after 32 weeks of incubation (data not shown).

Reductive debromination by a pyruvate enrichment culture. Hudson River microorganisms which had been transferred repeatedly on Aroclor 1242 and modified RAMM media containing pyruvate as an added electron donor and carbon source (Morris, this publication, Chapter 4) were evaluated for their ability to debrominate Firemaster. These studies were conducted in serum bottles, and in the presence of non-autoclaved, clean Hudson River sediment. At 16 weeks, the average number of *meta* and *para* bromines had decreased 17%, resulting in a decrease of 0.7 bromines per biphenyl molecule (data not shown). The major debromination products observed were 2,5-2',5'-BB, 2,4-2',5'-BB, 2,4-2',4'-BB, and 2,5-2'-BB (Figure 7). Also observed after 16 weeks of incubation were 2-2'-BB (Peak 4) and 2-BB (Peak 1). When week 16 samples were analyzed on a mass selective detector, biphenyl was detected at week 16 (Figure 8). Biphenyl was not observed in samples analyzed prior to week 16. This enrichment culture exhibited a shorter acclimation period and more debromination products than the Hudson River inoculum.

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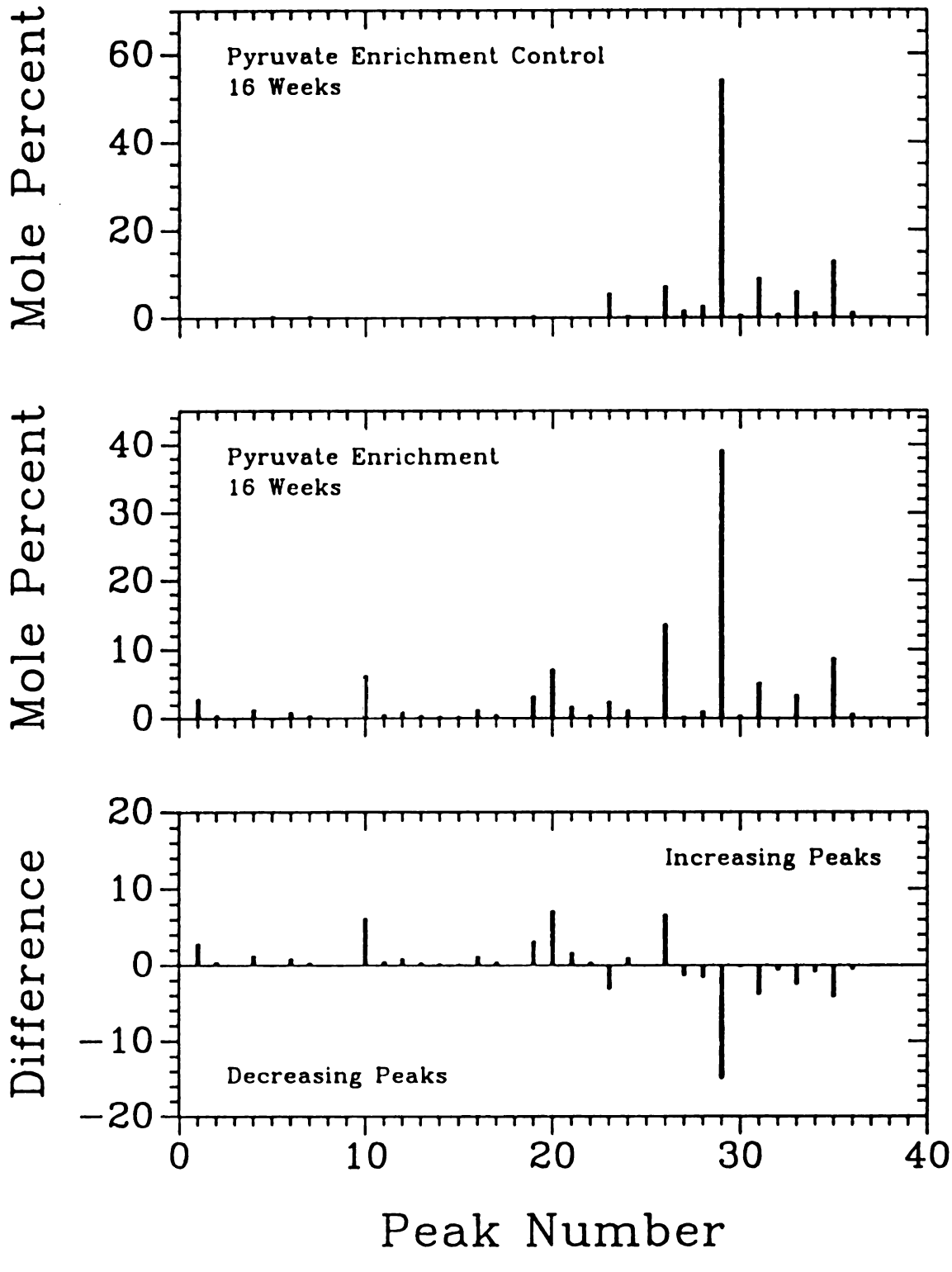

FIGURE 7. Mole percentage of PBBs represented by each chromatographic peak after 16 weeks of incubation with Hudson River microorganisms (previously transferred on clean Hudson River sediment). The lower histogram shows the mole percent increase and decrease in PBBs.

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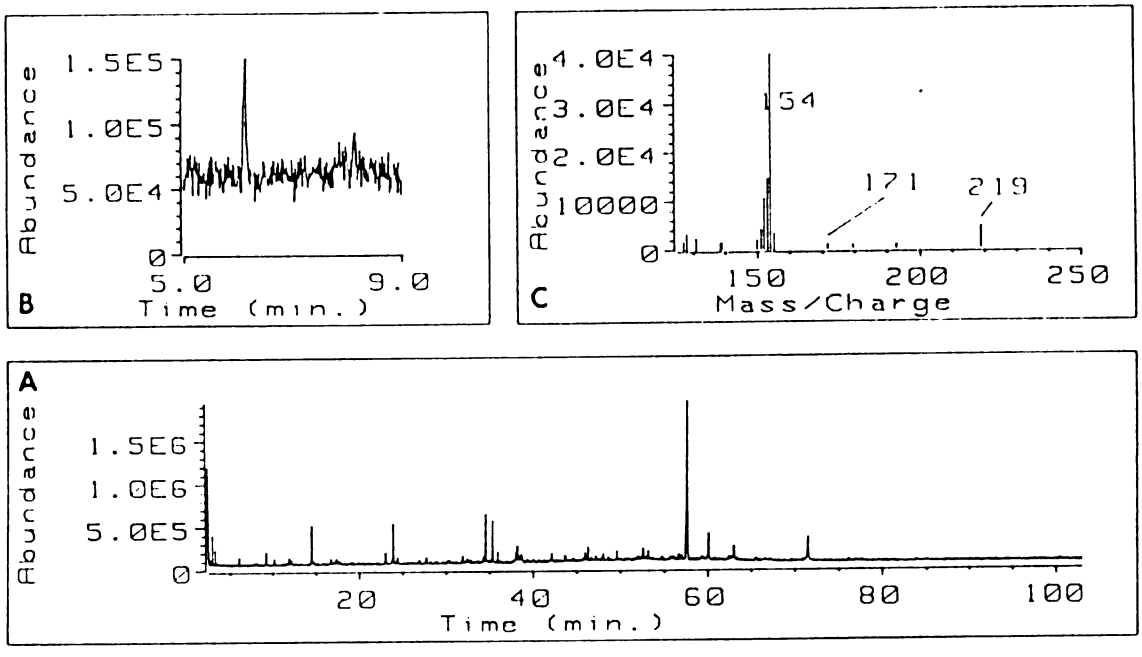

FIGURE 8. Gas chromatographic-mass spectrometric analysis of PBBs after 16 weeks of incubation with Hudson River microorganisms (previously transferred on clean Hudson River sediment). Panel A, total ion chromatogram (TIC) of PBBs in Firemaster after 16 weeks of incubation, panel B, detail of TIC region containing biphenyl; panel C, molecular ion spectra of TIC peak eluting at 6.136 min.

Discussion

Microorganisms from three sites, one contaminated with PBBs (Pine River) and two contaminated with PCBs (Hudson River and Silver Lake), were capable of debromination of the commercial PBB mixture, Firemaster, when incubated with reduced anaerobic mineral medium in the laboratory. Debromination activity occurred at both *meta* and *para* positions of the PBB congeners. It has been observed that the more heavily chlorinated PCB mixtures (e.g., Aroclor 1260) show an increased acclimation period prior to dechlorination by Hudson River microorganisms and yield less chlorine removal than less chlorinated PCB mixtures, such as Aroclor 1242 (Quensen et al., 1990). In the case of Firemaster, the acclimation period and extent of dehalogenation obtained with Hudson River inoculum was comparable to that previously observed with Aroclor 1260 (Quensen et al., 1990). When compared in a single study to microorganisms from PCB-contaminated sediments from the Hudson River and Silver Lake, Pine River microorganisms showed the highest PBB debromination activity (Figure 3). However, in an experiment set up at a separate time, Hudson River microorganisms showed comparable debromination activity (Figure 1). Apparently, experimental conditions such as the age of the inoculum can directly influence the degree of dehalogenation observed. The maximum extent of PBB debromination was about 1.25 (of six) bromines per biphenyl. Addition of microorganisms which had been previously exposed to Aroclor 1242 and pyruvate (a potential electron donor) decreased the acclimation period prior to debromination, and resulted in additional debromination products.

The extent of dehalogenation of Firemaster, Aroclor 1242, and Aroclor 1260 by microorganisms from the three study sites is summarized in Table 1. The highest *meta* plus *para* halogen removal at week 40 was 59% for Aroclor 1242 by Hudson

River microorganisms, increasing to 76% at week 73. This compares with an 85% removal within 12 weeks in our previous study (Quensen et al., 1990). The Pine River microorganisms resulted in the highest extent of Firemaster dehalogenation (32%) followed by the Hudson River inoculum which gave values of 29% and 12% in two separate experiments. The Silver Lake inocula removed 18% *meta* plus *para* chlorines from Aroclor 1260 compared to 19% in our previous study (Quensen et al., 1990). The Silver Lake inocula resulted in little (3%) or nodehalogenation of Firemaster. Thus, in each of the three experiments summarized in Table 1, the maximum extent of dehalogenation occurred when the inoculum was dehalogenating the same PBB or PCB mixture present in the inoculum source, i.e. the contaminated sediment. In general, organisms with prior exposure to a specific halogenated biphenyl mixture (e.g. Firemaster, Aroclor 1242, Aroclor 1260) seem to have a greater capability for dehalogenating that mixture (Quensen et al., 1990) than organisms exposed to other halogenated biphenyl mixtures. Microorganisms eluted from Pine River sediments upstream of the site of PBB contamination were not able to dehalogenate Firemaster or Aroclor 1242 (Morris, this publication, Chapter 2).

Silver Lake microorganisms, previously exposed to Aroclor 1260, a heavily chlorinated PCB mixture, showed little debromination of Firemaster. From previous observations, Silver Lake inoculum exhibited a shorter acclimation period and more rapid dechlorination of Aroclor 1260 than Hudson River microorganisms which had previous exposure to Aroclor 1242, indicating a greater capability to dechlorinate more heavily chlorinated PCB congeners (Quensen et al., 1990). In this study, Silver Lake inoculum supported very limited debromination activity on Firemaster in contrast to Hudson River inoculum (exposed to Aroclor 1242) and Pine River inoculum (exposed to Firemaster). These results suggest that the

dechlorination enzyme(s) of Silver Lake microorganisms are very specific, or that PBB uptake is more difficult for these microorganisms. As a result, previous exposure to Aroclor 1260 (PCB) did not predispose these microorganisms for optimal Firemaster (PBB) debromination.

The PBB mixture differs from the more complex PCB mixtures, with one congener, 2,4,5,-2',4',5'-BB, representing approximately 50% of the mixture. The dehalogenation products of Firemaster can be accounted for by the debromiantion of this congener. While roughly 33% of this congener was debrominated by Hudson River microorganisms when present as a component of the Firemaster mixture, when incubated alone no measurable debromination occurred during 32 weeks of incubation. This suggests differential behavior of the PBB congener when present as a component of the PBB mixture. Perhaps the presence of one or more of the other congeners could be required to elicit a response (e.g., enzyme induction) which triggers reductive dehalogenation. This observation does emphasize that the behavior of complex mixtures, and single components from these mixtures, may be quite different.

At a comparatively lower concentration of Firemaster (50 ppm), PBB dechlorination did not occur. In this instance the necessary congeners may be at too low a concentration to induce dehalogenation activity. The concentration dependence for reductive debromination of Firemaster has also been observed previously with PCB mixtures. Dechlorination of PCBs by Hudson River microorganisms has been shown to occur more extensively at higher concentrations (i.e 700 ppm) whereas at lower concentrations (i.e. 14 ppm) dechlorination was not observed (Quensen et al., 1988). A similar response has also been observed in sediments of Woods Pond (Lenox, MA) which is contaminated with Aroclor 1260. Bedard and colleagues (1990) observed only slight *meta* and *para* dechlorination of

endogenous PCBs in sediments of Woods Pond. However, slurries of Woods Pond sediment could be stimulated to dechlorinate endogenous PCBs by adding a high concentration of a single PCB. It has also been demonstrated that an anaerobic microorganism can obtain energy from reductive dehalogenation for growth (Dolfing, 1990; Mohn and Tiedje, 1990). It is unknown whether specific congeners in the PBB or PCB mxitures behave as dechlorination inducers or provide the dehaolgenating microorganisms energy for growth.

The detection of biphenyl as a debromination product of Firemaster in incubations with the pyruvate enrichment indicates the potential for complete debromination of some PBB congeners. There are no components of Firemaster without at least one *ortho*-substituted chlorine present. Three congeners, 2,4,5-3',4'-BB (peak 26), 2,4,5-3',4',5'-BB (peak 33) and 2,3,4,5-3',4'-BB (peak 34) are the only possible sources of 2-BB if debromination occurred strictly at the *meta* and *para* positions. Hudson River microorganisms accumulated 2,5-2'-BB and 2-2'-BB. Pine River microorganisms accumulated several tetrabromobiphenyls, trichlorobiphenyls, and a small amount of 2-2'-BB. The pyruvate enrichment with Hudson River microorganisms showed the accumulation of several tetrachlorobiphenyls. In addition, this enrichment resulted in the accumulation of 2,5-2'-BB, and small amounts of 2-2'-BB, 2-BB, and biphenyl. A possible pathway for the complete debromination of the major component of Firemaster to biphenyl would be the sequential debromination of 2,4,5-2',4',5'-BB to 2,4-2',5'-BB and 2,5-2',5'-BB, to 2,5-2'-BB, to 2-2'-BB, to 2-BB, and potentially to biphenyl (Figure 9). While we did not previously report PCB dechlorination from the *ortho* positions, there is *in situ* evidence of *ortho* dechlorination of Aroclor 1260 in sediments from Silver Lake (Brown et al., 1987a). And in the laboratory, *ortho* and *meta* chlorine removal from a single PCB congener, 2,3,5,6-CB, during 37 weeks of incubation with

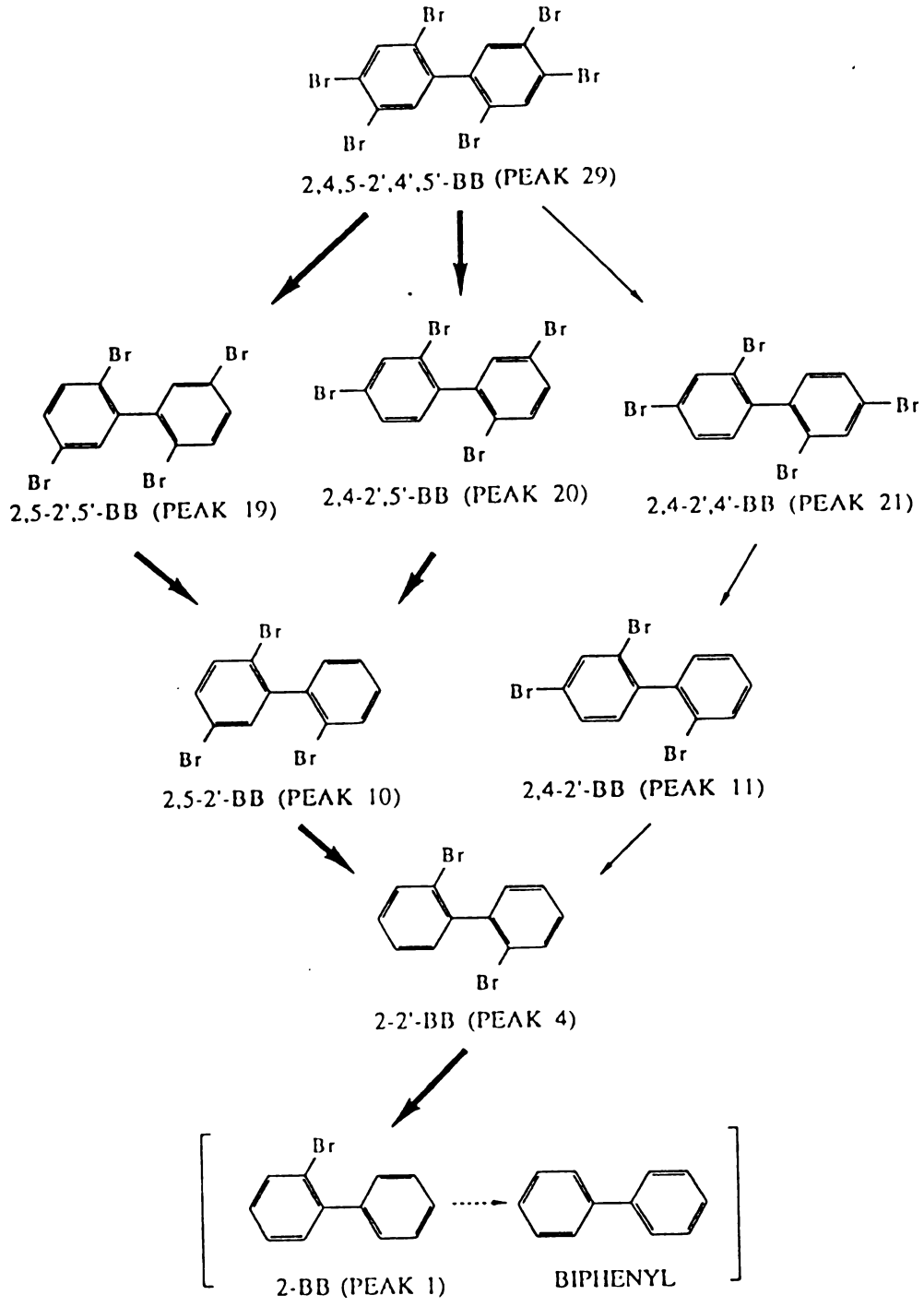

FIGURE 9. Proposed pathway for the reductive debromination of 2,4,5-2',4',5'-hexabromobiphenyl by Pine River and Hudson River sediment microrganisms.

methanogenic sediment from Woods Pond has been observed (Van Dort and Bedard, 1991).

Microorganisms eluted from PBB-contaminated sediments (Firemaster) and PCB-contaminated sediments (Aroclor 1242) were capable of reductive debromination of the commercial PBB mixture, Firemaster. The decrease in acclimation period prior to debromination and the presence of additional debromination products observed when the pyruvate enrichment was incubated with Firemaster, suggests the ability to optimize reductive dehalogenation of heavily halogenated biphenyls. Although aerobic PCB degradation of Aroclor 1260, averaging 6 chlorines per biphenyl molecule, has not been observed (Abramowicz, 1990), dechlorination of the mixture would enhance the potential for aerobic biodegradation. In this study, an average of 1.25 bromines per biphenyl were removed by Pine River microorganisms. This compares to 0.75 chlorines per biphenyl removed from Aroclor 1260 by Silver Lake microorganisms and 1.5 chlorines per biphenyl removed from Aroclor 1242 by Hudson River microorganisms (Quensen et al., 1990). Like PCBs, PBBs were considered to be highly recalcitrant in anaerobic environments. However, reductive dehalogenation of PBBs, shown to occur under strict anaerobic conditions in the laboratory, may provide a route to PBB bioremediation in natural environments.

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Chapter 4:

Establishment of a PCB-degrading enrichment culture with predominately *meta*-dechlorination

The following was collaborative work with Dr. William W. Mohn.

Introduction

Polychlorinated biphenyls (PCBs) are a group of stable industrial chemicals that consist of complex mixtures known as Aroclors. These PCB mixtures have been considered highly persistent in the environment. Altered PCB congener distribution patterns were observed in upper Hudson River sediment samples, and proposed to be the result of a biologically mediated process (Brown et al., 1984; Brown et al., 1987a; Brown et al., 1987b). This was later substantiated when Quensen et al. (1988) demonstrated that microorganisms eluted from PCB-contaminated Hudson River sediments were capable of reductive dechlorination of the PCB commercial mixture, Aroclor 1242, in the presence of non-contaminated sediments. Reductive dehalogenation has now been observed with four of the commercial Aroclor mixtures which differ in the percentage of chlorine by weight (Quensen et al., 1990).

Reductive dehalogenation is the only known biodegradation process for the highly chlorinated PCB mixtures, including several significant environmental contaminants (Mohn and Tiedje, 1992). Thus, the process is of great potential importance in developing biotreatment processes for halogenated compounds. But in nearly all reported cases, the dehalogenation of aromatic compounds occurs in undefined cultures (Mohn and Tiedje, 1992). Attempts to enhance dehalogenation rates, as by the addition of organic carbon sources (Nies and Vogel, 1990), have led to ambiguous and sometimes conflicting results, in part because they were made with defined cultures. Our basic understanding of the process would greatly increase if more interpretable experiments could be conducted with pure cultures (Mohn and Tiedje, 1992).

Pure cultures capable of reductive dehalogenation of aromatic compounds, however, have been difficult to obtain, perhaps because dehalogenation is usually associated with syntrophic communities. Only one obligate anaerobe has been isolated which is capable of aryl reductive dehalogenation, *Desulfomonile tiedjei*

(Shelton and Tiedje, 1984; DeWeerd et al., 1990); this organism is capable of dehalogenating certain benzoates (Shelton and Tiedje, 1984; Cole and Tiedje, unpublished data), phenols (Mohn and Tiedje, 1992) and ethylenes (Fathepure et al., 1987). Importantly, *D. tiedjei* is able to gain energy for growth by coupling electron transport to reductive dechlorination (Dolfing and Tiedje, 1987; Dolfing, 1990; Mohn and Tiedje, 1990; Mohn and Tiedje, 1991).

In this study, we attempted to enrich and transfer PCB dechlorinating microorganisms using PCBs as the electron acceptors. We used culture conditions found to be favorable for aryl dechlorination by *Desulfomonile tiedjei* (J.R. Cole, unpublished data; Apajalahti et al., 1989; DeWeerd et al., 1990; Mohn and Tiedje, 1990). We also examined the effects of several electron donors and acceptors on the dehclorinating activity of this culture. A stable, transferable, PCB-dechlorinating culture was established. The culture is sediment-dependent, methanogenic and preferentially removes *meta*-chlorines from the commercial PCB mixture, Aroclor 1242.

Materials and Methods

Sediments. Aroclor 1242-contaminated sediments were collected (August 1988) from the upper Hudson River at River Mile 193.5 near Hudson Falls, N.Y. (site H7 in Brown et al., 1984). Sediments were collected with a post hole digger (to a depth of approximately 25 cm) and transported to the laboratory in tightly sealed metal cans. In the laboratory, PCB-contaminated sediments were placed in 2 liter Erlenmeyer flasks, which had been flushed with N_2-CO_2 (80:20). Equal volumes of sediment and medium were placed in flasks under a N_2-CO_2 atmosphere and sealed with butyl rubber stoppers. Supernatant from these sediment slurries was used as the primary inoculum source. Non-PCB contaminated sediments were

collected upstream at River Mile 205 (Spiers Falls). Non-contaminated sediments were air-dried and sieved through a 2mm screen prior to use.

Medium. The mineral medium of Shelton and Tiedje (1984) was used with the following modifications and additions: 1 mM cysteine instead of Na_2S, 10 mM instead of 4 mM potassium phosphate buffer (pH 7.0), $NaHCO_3$ omitted, vitamins and 0.1 mM titanium (III) citrate were added. Vitamins, titanium (III) citrate and cosubstrates (where indicated) were added after autoclaving from filter-sterilized stock solutions to the autoclaved mineral medium. Ferric oxyhydroxide was prepared according to Lovely and Phillips (1986). Vitamins included a mixture of vitamins generally required by various anaerobes (Wolin et al., 1963) plus 450 μg/l nicotinamide and 200 μg/l naphthoquinone (DeWeerd et al., 1990). Titanium (III) citrate was prepared according to Zehnder and Wuhrmann (1976). The gas phase was N_2 unless otherwise indicated. The pH was 7.0 before autoclaving.

Incubation conditions. The experimental protocol used 160-ml serum bottles containing 25 g of non-PCB-contaminated sediments (not autoclaved). While the bottles were being flushed with O_2-free N_2, 40 ml of anaerobic mineral medium containing 20 mM pyruvate (or other amendment) and 10 ml of inoculum were added. For the first transfer (primary inoculum), the sediment-mineral medium slurry was shaken and allowed to briefly settle before withdrawing 10 ml of inoculum. Succeeding transfers followed the same shaking then settling procedure. After inoculation, a 20% (wt/vol) solution of Aroclors (Monsanto Co., St. Louis, MO) in acetone was added to each serum bottle resulting in a final Aroclor concentration of 1 mg/g sediment. Serum bottles were shaken and then incubated at 25^{o}C in the dark. Anaerobic conditions were maintained during transfers. All data are means from duplicate incubations; the standard error in these types of assays is generally less than 10% as illustrated previously (Quensen et al., 1990).

Analyses. Just prior to sampling PCBs, headspace gases (CO_2, CH_4) were sampled and quantified with a Carle Model AGC-111 gas chromatograph equiped with a 6 m Porapak Q column and microthermistor detector with Argon as the carrier gas (20 ml/min). Organic acids present in the liquid culture medium were analyzed by high pressure liquid chromatography (Stevens et al., 1988) with the column temperature at 60°C. Sediments were sampled, extracted and analyzed for individual PCB congeners by capillary gas chromatography with an electron capture detector as described previously (Quensen et al., 1990). Reductive dechlorination was evaluated by observing the decrease in the average number of *meta*- and *para*-chlorine substituents per biphenyl molecule, as described in Quensen, et al. (1990). For a list of PCB congeners represented by each chromatographic peak analyzed, refer to Table 1.

Isolation. H_2-using organisms were isolated from cultures on pyruvate plus Aroclor 1242 medium after the seventh transfer. The above-described basal medium (with vitamins and reductants, but without Aroclor 1242) was used in roll tubes and was solidified with 1% nobel agar (Difco, Detroit, MI). A gas phase of 2 atm H_2-CO_2 (80:20) was used. In addition, the above medium was also made with 1 mM bromoethane sulfonate (BESA) and with 5 mM Na_2SO_4. Isolates were maintained on the respective media without agar.

Test for PCB dechlorination by pure cultures. The following four stock cultures were tested: *Desulfomonile tiedjei* DCB-1, *Methanobacter* sp. DG1, *Methanospirillum* sp. PM1 and *Methanosarcina* sp. MS. The following six isolates from the enrichment were also tested: 1 rod isolated on H_2-CO_2, as well as 1 rod, 1 coccus and 3 vibrios isolated on H_2-CO_2 plus sulfate. The above cultures in late-log phase were separately used as inocula (20%) for pyruvate plus Aroclor 1242 medium which included non-contaminated sediment. Each isolate was tested in the

TABLE 1. Polychlorinated biphenyl (PBB) structure associated with each chromatographic peak.

Peak #	Structure
1	2
2	4
3	2-2 26
4	24 25
5	2-3
6	2-4 23
7	26-2
8	34 3-4
9	25-2 4-4
10	24-2
11	26-3 236
12	23-2 26-4
13	35-2 (26-26)
14	245
15	25-3
16	24-3
17	25-4
18	24-4 (246-2)
19	34-2 234 23-3 25-26
20	23-4 (24-26)
21	236-2
22	23-26
23	25-25 26-35
24	24-25
25	24-24
26	245-2 246-4
27	34-3
28	23-25
29	23-24 236-3 34-4
30	26-34 234-2 236-4
31	236-26
32	23-23
33	235-3 245-3 246-24
34	23-35 235-4
35	245-4 (235-26)
36	25-34 345-2
37	236-25 245-26 24-34
38	234-3 236-24
39	23-34 234-4
40	245-25 235-24
41	245-24
42	236-246 2356-3 246-34
43	245-23 2345-2
44	234-25 2346-4 235-35
45	234-24
46	236-236
47	34-34 236-34
48	2356-25
49	235-236 345-25
50	245-34 236-245 2345-3
51	2356-23 2345-26
52	345-23 2346-23
53	235-245 2346-35
54	245-245
55	234-236 234-34
56	2345-25
57	2356-236
58	2345-24
59	2346-236
60	234-245 2356-34
61	2346-34
62	2356-235
63	2346-235
64	2356-245 2345-24
65	2346-245
66	245-345
67	23456-25
68	2345-236 23456-24
69	2356-234
70	2346-234 2345-34
71	23456-23 2346-2356
72	2345-235 23456-35
73	2345-245
74	2356-345
75	2346-345
76	23456-236
77	2345-234
78	23456-34
79	2356-2345
80	2345-2346 23456-245
81	2345-345
82	23456-234
83	23456-2356
84	2345-2345
85	23456-345
86	23456-2345
87	Internal Standard
88	23456-23456

presence of both sterile and non-sterile sediments. The sediments were sterilized by autoclaving for 1 h on three successive days. Each test was performed in duplicate.

Results

PCB-dechlorination by serially transferred cultures. The original inoculum was initially grown in four media: (1) no cosubstrate (except as provided by the non-contaminated sediment), (2) 10 mM sodium formate, (3) 10 mM sodium formate plus 1 mM bromoethanesulfonate (to prevent electron flow to methanogenesis), and (4) 20 mM sodium pyruvate. Dechlorination of Aroclor 1242 was not observed in any treatment until after 8 weeks. Cultures with no cosubstrate and those with pyruvate had the highest rates of dechlorination at week 12 (Figure 1); only these two media were used for the second transfer. The transferred cultures with pyruvate had a much higher dechlorination rate than those with no added co-substrate (Figure 1). Additionally, in transferred cultures containing pyruvate, dechlorination occurred much earlier, within the first 4 weeks (Figure 1).

In subsequent serial transfers, only the pyruvate medium was used because it provided the most effective dechlorination. Cultures were transferred seven more times on pyruvate plus Aroclor 1242 medium over a period of 12 months. Dechlorination activity was maintained throughout, although the extent of dechlorination decreased from about 0.9 mol chlorine/biphenyl removal in the first three transfers to about 0.5 mol chlorine/biphenyl removal in the later transfers (Table 2). The estimated rate of dechlorination was 18 μg atom chlorine released l^{-1} d^{-1} for the later transfers. The pattern of dechlorination observed with these transfers remained consistent, and resembles pattern M (Brown et al., 1989), which is characterized by preferential removal of chlorines from the *meta* position of the biphenyl molecule (Figure 2). *Para*-chlorines were more resistant to dechlorination, as shown by little change in peaks 6 (2,4-CB, 2,3-CB), 10 (2,4-2-CB) and 18 (2,4-4-

Table 2. PCB chlorine-content reduction at week 4 by successive serial transfers of the pyruvate-Aroclor 1242 enrichment culture.

Transfer	*Meta* and *para* Cl/biphenyl	Total Cl/biphenyl
Autoclaved Control	1.79	3.20
1[a]	0.93	2.34
2	0.81	2.27
3	0.99	2.62
4	1.45	2.88
5	1.26	2.70
6	1.12	2.58
7	1.31	2.78
8	1.34	2.81
9	1.38	2.84

[a]Data for week 12 in the original enrichment because no dechlorination was observed at weeks 4 and 8.

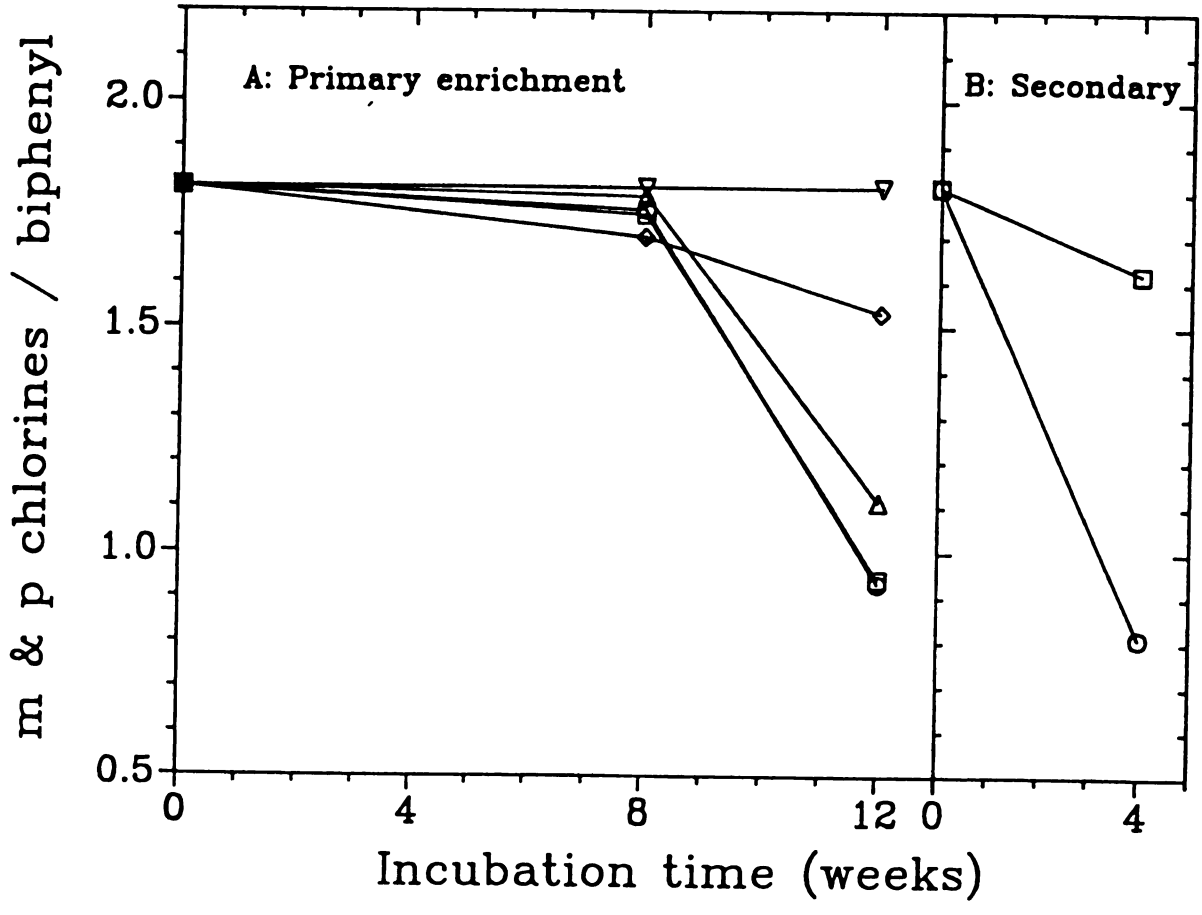

FIGURE 1. Dechlorination of Aroclor 1242 by microorganisms eluted from Hudson River sediments. (A) Primary enrichment cultures grown in the presence of no co-substrate (squares), 20 mM pyruvate (circles), 10 mM formate (triangles), 10 mM formate plus 1 mM BES (diamonds), and an autoclaved control (inverted triangles). (B) Secondary transfer of the no co-substrate (squares), and 20 mM pyruvate (circles) primary enrichment cultures.

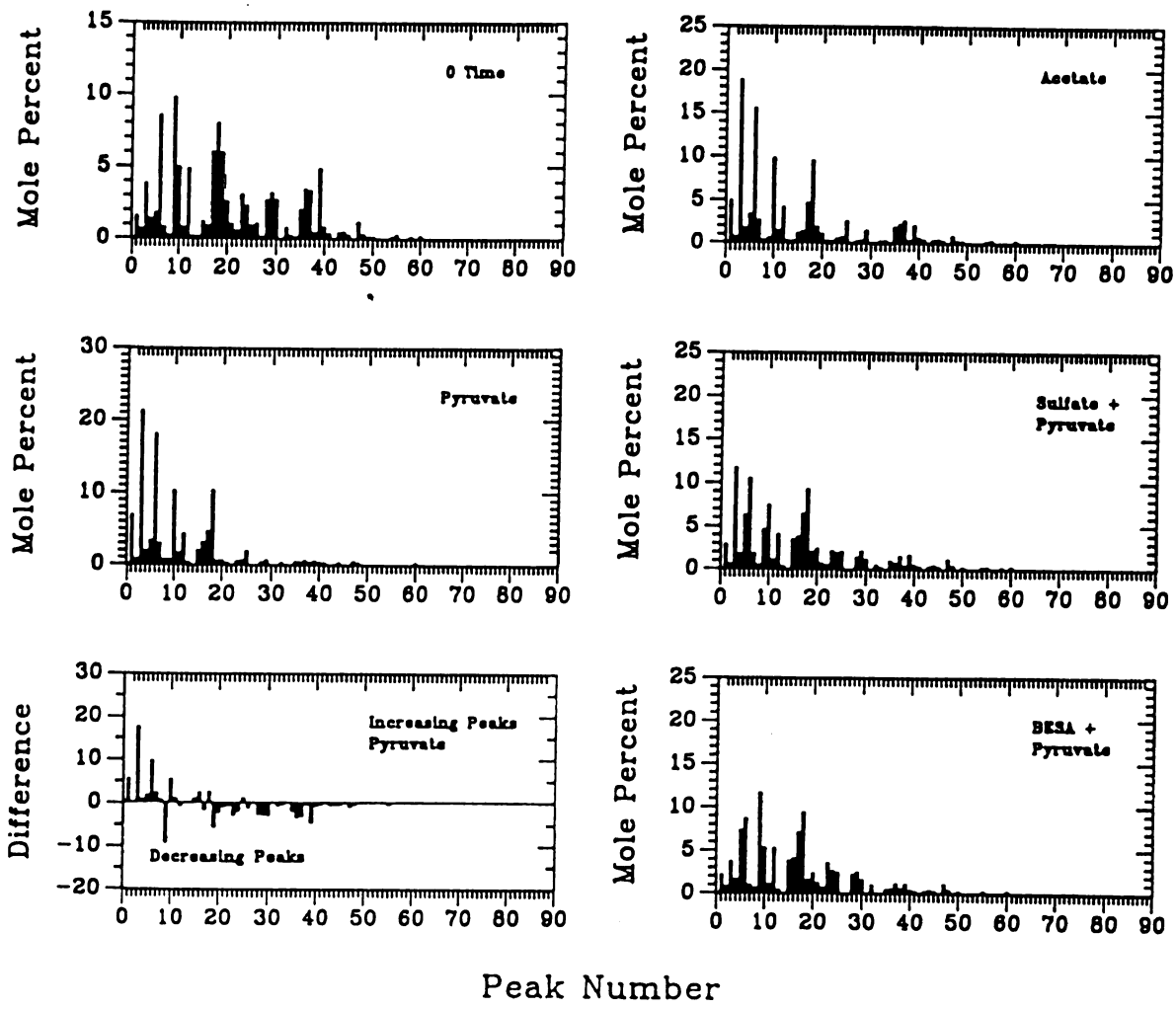

FIGURE 2. Mole percentage of Aroclor 1242 congeners after incubation with the enrichment culture under the indicated condition. The left-hand panels show Aroclor 1242 at zero-time and its products after 4 weeks of incubation with pyruvate. The right-hand panels show Aroclor 1242 products after 12 weeks of incubation with acetate instead of pyruvate as the electron donor, with sulfate plus pyruvate, and with BESA plus pyruvate. The identities of congeners by peak number can be found in Table 1.

CB) (Figure 2). To determine if electron donor was limiting further dechlorination in the enrichment, some bottles that were not transferred were amended with pyruvate at monthly intervals for three months; the extent of dechlorination did not increase.

The enrichments described here included non-PCB-contaminated sediments because previous experience has shown that they are necessary for significant PCB dechlorination. The sediments used contained an indigenous population that did not dechlorinate PCBs during the four week incubation between transfers of these cultures (e.g., Figure 1). This indigenous population did not appear essential for PCB-dechlorination, since autoclaving the non-contaminated sediments for 20 min on three successive days did not affect PCB dechlorination (data not shown).

The inoculum concentration made little difference in the rate and extent of PCB dechlorination; 20%, 10% 5%, and 1% inocula taken from the third transfer all showed similar dechlorination after four weeks, and the 0% inoculum showed no dechlorination. Thus, the inoculum apparently contained a PCB-dechlorinating population(s) at sufficient density so that a 1% inoculum was not limiting.

The patterns of pyruvate metabolism and PCB dechlorination were compared in cultures inoculated with the pyruvate-Aroclor 1242 enrichment (Figure 3). Pyruvate does not appear to be the immediate electron donor for dechlorination because it was completely consumed before dechlorination began. Acetate appeared as a transient product, and its consumption coincided with the onset of methane production and with dechlorination.

Effect of electron donors and acceptors. Potential electron acceptors and bromoethane sulfonate were added to cultures inoculated with the pyruvate plus Aroclor 1242 enrichment culture (Figure 4A). Sulfate and BESA reduced dechlorination activity by 50% compared to the CO_2 treatment, ferric oxyhydroxide reduced dechlorination by 12%. In the presence of carbon dioxide and nitrate (10

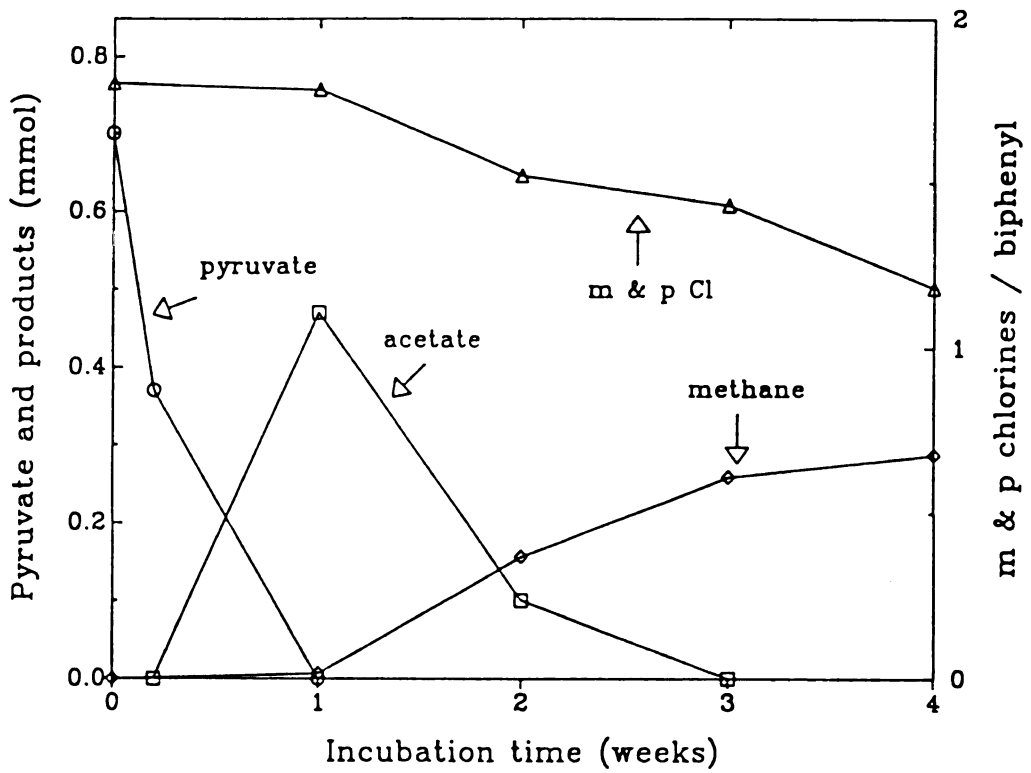

FIGURE 3. Temporal pattern of pyruvate metabolism and dechlorination by the pyruvate plus Aroclor 1242 enrichment culture.

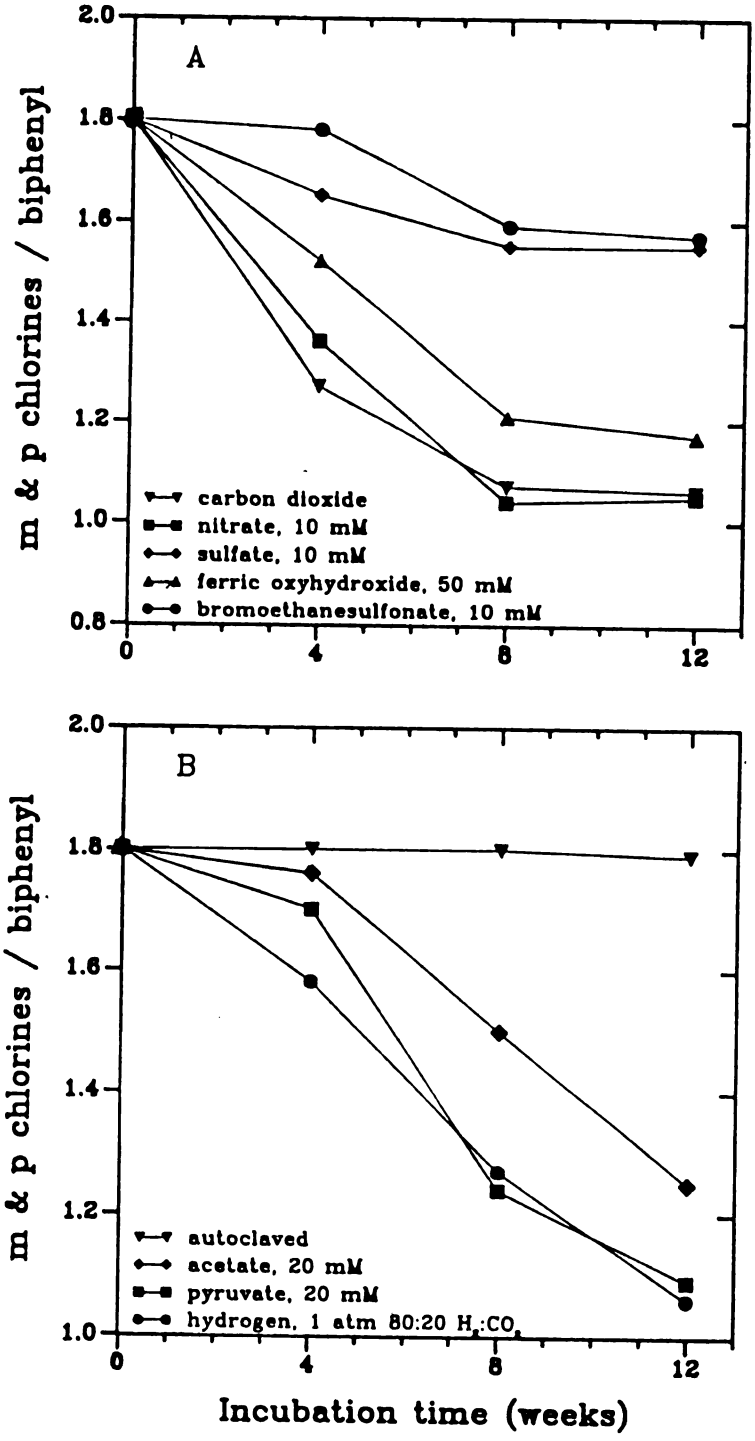

FIGURE 4. Effect on Aroclor 1242 dechlorination by the pyruvate-Aroclor 1242 enrichment by (A) potential electron acceptors and (B) electron donors. Carbon dioxide addition was 30 mM $NaHCO_3$ plus 20% CO_2 in headspace. Inocula were from the secondary transfer. The electron donor and electron acceptor studies were not established simultaneously.

mM), dechlorination rates were the highest, and comparable. Some CO_2 was present in all treatments since it was a product of pyruvate metabolism. The amounts of mono- and di-chlorinated PCBs produced were reduced in the sulfate and particularly the BESA treated cultures (Figure 2).

Hydrogen and acetate were compared to pyruvate as electron donors for dechlorination by the enriched inoculum (Figure 4B). All three substrate additions resulted in equivalent dechlorination rates, but the onset of dechlorination was delayed about 4 weeks when acetate was the substrate. All electron donor and acceptor treatments produced similar congener profiles to those shown for the pyruvate addition (Figure 2), except for the three treatments shown in Figure 2. In all cases, the major PCB products were represented by peaks 1,3,6,10,18 and 25. These are characteristic of pattern M dechlorination.

Examination of dechlorination by pure cultures. H_2 was chosen as the electron donor for isolation instead of pyruvate because H_2 stimulated dechlorination equally well in the pyruvate enrichment (Figure 4B), and would eliminate the many pyruvate fermentors found in such enrichments (Tiedje et al., 1992). The dominant H_2-using isolates would most likely be those most enriched by the dechlorination selection regimen; thus representatives of the most numerous colony types were picked. Six H_2-using isolates were obtained and tested, including a methanogen (presumably) and five sulfate reducers. Four cultures from our culture collection, including a sulfate reducer and three methanogens were also tested. We were unable to isolate acetogens from the pyruvate plus Aroclor culture on H_2 plus BESA medium.

All 10 pure cultures tested were unable to dechlorinate Aroclor 1242 during 16-week incubations under the test conditions. These conditions included the pure culture plus the indigenous community of the non-contaminated sediments, and the pure cultures in the same sediments which had been sterilized.

Dechlorination of various Aroclors. The dechlorination of Aroclors 1221, 1242, 1248, 1254, and 1260 was examined using transfers of the pyruvate plus Aroclor 1242 culture with 5% inocula (vol/vol) compared to cultures with only the indigenous organisms present in the non-contaminated sediment. The inoculum caused more rapid dechlorination of four of the five Aroclors tested (Figure 5). Aroclor 1221 was not dechlorinated in either case, probably because it is primarily biphenyl, 2-CB, and 4-CB, which are terminal products of pattern M dechlorination. While methane was observed in the presence of Aroclors 1242, 1248, 1254, and 1260 by week four, the addition of Aroclor 1221 caused up to an eight week lag in detectable methane formation. Surprisingly, Aroclor 1254 was more extensively dechlorinated than either Aroclor 1242, the enrichment substrate, or Aroclor 1248, which has many of the same congeners as Aroclor 1242. Some dechlorination of Aroclor 1260 also occurred.

Discussion

A PCB-dechlorinating community was enriched and maintained by serial transfer on a pyruvate-Aroclor-sediment medium. The enrichment occurred primarily during the first and second transfers while later serial transfers showed no apparent increase in selection as evidenced by the rate of dechlorination. Growth of the dechlorinating organisms must have occurred to maintain the activity through the nine serial transfers and to have equal activity in the serial transfer with a 1% inoculum. Evidence for at least some selection of PCB-dechlorinators is shown by the much earlier onset of dechlorination of four Aroclors when inoculated with the enrichment compared to that observed with the indigenous sediment organisms. The lack of further selective enrichment during serial transfer means that this method alone is inadequate to enrich a community of predominately dechlorinating organisms.

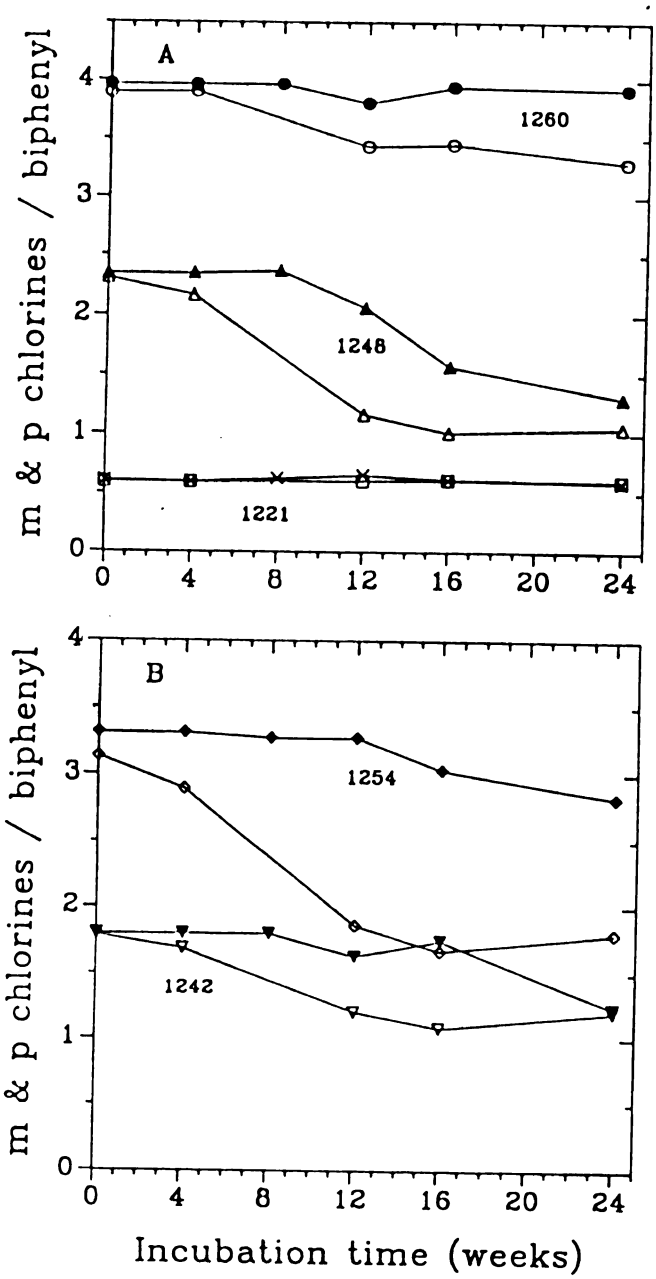

FIGURE 5. Dechlorination of five Aroclors when inoculated with the pyruvate-Aroclor 1242 enrichment culture (open symbols) compared to no inoculant addition other than that supplied by non-PCB contaminated Hudson River sediments (closed symbols). Aroclor 1260 (circles), Aroclor 1254 (diamonds), Aroclor 1248 (diamonds), Aroclor 1242 (inverted diamonds), and Aroclor 1221 (squares for inoculant addition, asterik for no inoculant addition).

Sediments were required for dechlorination activity in the pyruvate plus Aroclor 1242 medium. The role of the sediment is unknown, but it does disperse the poorly water soluble PCBs and it probably provides some carbon and growth factors for the microbial community. The sediment requirement was not specific to sediments from the Hudson River. Other sediments, surface soils and solid materials have also supported dechlorination activity (Griffith et al., 1990; Morris, this publication, chapter 5). Although non-sterile, non-PCB-contaminated Hudson River sediments were used for the serial transfers in this study, the use of sterile sediments resulted in comparable dechlorination activity.

Neither the six dominant H_2-consuming isolates from the pyruvate-Aroclor enrichment, nor four other pure cultures of anaerobes dechlorinated PCBs under the same assay conditions that successfully supported dechlorination by the enrichment. This group of ten isolates included six sulfate reducers and four methanogens, which are physiological groups previously shown to contain other dechlorinating activities (Mohn and Tiedje, 1992). Dechlorination by the enrichments in this study was also inhibited by sulfate and BESA which is consistent with the potential involvement of these physiological groups in dechlorination. While this is not an extensive screening of the many organisms probably present in this community, it is a reasonable sampling of the more dominant and likely dechlorinating types. Furthermore, it was a test under conditions known to support dechlorination, and did include testing isolates under conditions that provided potential consortia partners (non-sterile sediments) if they were essential, as well as in the absence of competitors for electron donors and growth factors (sterile sediments). Strategies other than a standard screening of isolates will likely be necessary to identify PCB-dechlorinating organisms.

The availability of electron acceptors in anaerobic communities will likely affect reductive dehalogenation, both because these compounds might compete with

halogenated compounds for reducing potential, and because these compounds will impose different selective pressures on growing communities. In this study, sulfate clearly inhibited dehalogenation. Several other reports conclude that reductive dehalogenation by anaerobic communities is inhibited by sulfate (Gibson and Suflita, 1986; Beeman and Suflita, 1987; Sharak-Genthner et al., 1989a and 1989b; Adrian and Suflita, 1990; Kuhn et al., 1990) and BESA (Sharak-Genthner et al., 1989). However, these inhibitory effects are not universal, as exceptions to the above findings exist (Bosma et al., 1988; Haggblom et al., 1990). The use of undefined cultures limits furthur understanding of these effects.

BESA, known to block electron flow to CH_4, was also inhibitory. In the experiments reported here and in earlier studies (Nies and Vogel, 1990; Quensen et al., 1988; Quensen et al., 1990), PCB dechlorination was always concomitant with methanogensis. We, therefore, interpreted the BESA effect as evidence that either methanogens directly catalyzed dehalogenation or that they were essential to an obligatory syntrophic community which furnished the necessary physiochemical environment for the dehalogenating microorganisms. However, we have since obtained pattern M dechlorination of Aroclor 1242 in the absence of methanogenesis (Ye et al., 1992) and now consider it more likely that BESA, an organohalide, directly inhibits dehalogenation.

Several dechlorination patterns have been observed in laboratory dechlorination studies which show preferences for *meta*-, *para*-, or *meta*- plus *para*-chlorine removal. Quensen et al. (1990) observed *meta*- plus *para* dechlorination (pattern C) when Hudson River microorganisms were added to Aroclor 1242 amended non-PCB contaminated sediment and a reduced anaerobic mineral medium. Dechlorination pattern C is characterized by the accumulation of 2-CB and 2-2'-CB and/or 2,6-CB (coeluting isomers) as the only major dechlorination products. This pattern appears to be the result of two different dechlorination

activities, perhaps mediated by different microorganisms (Quensen et al., 1990). Our enrichment exhibits mainly *meta* dechlorination (pattern M), while the primary culture dechlorinated at both the *meta* and *para* positions (pattern C). The enrichment culture accumulated not only 2-CB and 2-2'-CB and/or 2,6-CB, but also the *para*-substituted congener 2-4-CB. Additionally, 2,4-CB, 2,4-2'-CB and 2,4,-4'-CB showed no decrease due to dechlorination. This dechlorination pattern was stable over all transfers. The addition of pyruvate at monthly intervals to old cultures did not influence the cultures ability to further remove *para* chlorines, further suggesting that this enrichment is primarily *meta* dechlorinating microorganisms.

A different type of meta-dechlorinating community has been enriched from Hudson River sediment by Ye et al. (1992). This community was obtained by three serial transfers of pasteurized or 50% ethanol treated inocula that had been cultured on sterile sediment plus Aroclor 1242. The active dechlorinators in this case are thought to be spore-formers. The pasteurized enrichment cultures also carried out PCB-dechlorination in the absence of any methanogenesis. Because the meta-dechlorination occurs in these two different communities and is the most commonly observed, it may be more robust or broadly distributed among microbial groups.

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Chapter 5:

Sediment and Sediment Modification Influences on Reductive Dechlorination

Introduction

The ability of the anaerobic microbial consortium eluted from Hudson River sediments to dechlorinate PCBs has been dependent on the presence of sediments. Currently, researchers do not know whether this dependence is due to: 1) a nutrient supplied by the sediment (e.g. specific carbon sources, metals), 2) the sorption of PCBs to sediment surfaces which may increase PCB bioavailability or decrease PCB toxicity, or 3) a combination of the above. The low water solubility of individual PCB congeners in Aroclor mixtures (Hutzinger et al., 1974) could be a limiting factor in reductive dechlorination, due to limited bioavailability of PCB congeners. Water solubility enhancements of insoluble organic solutes, including certain PCB congeners (e.g. 2,4,5,2',5'-CB), has been shown to occur by partition-like interactions with dissolved organic matter (Chiou et al., 1986).

The purpose of the experiments described in this chapter was to help define the role of the sediment for the dechlorinating culture and try to find alternative, more well-defined supports for the reductive dechlorination of PCBs.

Materials and Methods

Medium. The mineral salts medium of Shelton and Tiedje (1984) was used with the following modifications: 1 mM cysteine instead of Na_2S, 10 mM instead of 4 mM potassium phosphate buffer(pH 7.0), $NaHCO_3$ omitted, 0.1 titanium (III) citrate, and vitamins (Wolin et al., 1963; 450 μg/l nicotinamide and 200 μg/l naphthoquinone, DeWeerd et al., 1990). Sodium pyruvate was added at a final concentration of 20 mM from sterilized stock solutions.

Cultures. All studies were conducted in 160 ml serum bottles, which had been flushed with O_2-free N_2 gas using a Hungate apparatus. Clean Hudson River sediment (25 g, non-sterile) was added to serum bottles that were subsequently amended with various other solid supports, anaerobic mineral medium (47.5 ml),

and the anaerobic inocula (2.5 ml). No preincubation period prior to inoculation was used. Since the studies were conducted over a long period of time, the inocula sources were different; however, all inocula were from pyruvate-plus-PCB transfer cultures which were enriched for *meta* dechlorination (refer to this publication, Chapter 4). A 20% (wt/vol) solution of Aroclor 1242 (Monsanto Co., St. Louis, MO) in acetone was added to each bottle to a final concentration of 1 mg/g sediment. Serum bottles were incubated at 25^{o}C in the dark.

Sediments and alternative solid supports. Hudson River sediments (non-PCB-contaminated) were collected at River Mile 205 near Hudson Falls, NY., and then were air-dried and sieved. Ashed sediments were prepared by heating air-dried sediments at 550^{o}C for 12 h in a muffle furnace (BLUE-M Electric Company, Blue Island, IL.) to remove organic carbon. Separation of the sand and silt plus clay fractions of Hudson River sediment was performed by sonicating (Heat Systems-Ultrasonics, Inc.) a 1:1 (v/v) sediment:deionized water slurry. This was followed by seiving (U.S.A. Standard Testing Sieve, No. 270, Soil Test, Inc.) to separate the sand from the silt plus clay. Particle size analysis of surface soils and sediment was performed by the hydrometer method (Michigan State University Soil Testing Laboratory, E. Lansing, MI). Total organic carbon was determined by CO_2 released from combustion (analysis by Huffman Laboratories, Golden, CO). Humic acid (sodium salt, Aldrich Chemical Co.), activated carbon (200 mesh, Fisher Scientific Co.), sand (standard Ottawa, EM Industries, Inc., Cherry Hill, N.J.), and Chromosorb W (acid washed, 120/140 mesh, Alltech Associates, Inc., Deerfield, IL) were used as amendments. Humic material was extracted from non-PCB contaminated Hudson River sediments with 0.1N NaOH, then neutralized, and the resulting freeze-dried precipitate used as an amendment (Schnitzer, 1982).

Analyses. PCBs were sampled, extracted, and analyzed according to Quensen et al.(1990) by capillary gas chromatography equipped with an electron capture detector.

Results and Discussion

Previous studies on the reductive dechlorination of Aroclor 1242 have been conducted in the presence of clean Hudson River sediments (Quensen et al., 1988, Quensen et al., 1990; this publication, Chapter 4) or PCB-contaminated Hudson River sediments (Nies and Vogel, 1990). Three different surface soils (silty clay loam, sandy loam and clay loam) were tested for their ability to support reductive dechlorination by anaerobic microorganisms from Hudson River sediments (Figure 1, Table 1). All three soils were able to support reductive dechlorination of Aroclor 1242 by Hudson River microorganisms, with dechlorination occurring primarily in the first 8 weeks of incubation. Non-PCB contaminated Hudson River sediment however, still supported the highest degree of dechlorination.

Varying amounts of clean Hudson River sediments (0.5, 1, 5, 10, 25, and 50 g) were added to serum bottles (160 ml) which contained a constant volume of liquid medium (47.5 ml), anaerobic inoculum (2.5 ml), and Aroclor 1242 (25 mg per serum bottle) (Figure 2). No dechlorination was observed in the presence of 0.5 and 1 g sediment, while the highest amount of sediment present was optimum for dechlorination activity.

The influence of the different sediment fractions on reductive dechlorination of Aroclor 1242 by Hudson River microorganisms was also studied. When clean Hudson River sediment was fractionated into the sand and silt plus clay fractions (with native organic matter present), 2.5 g of the silt plus clay fraction (8.09% total carbon) supported approximately the same amount of dechlorination over 32 weeks as did 22.5 g of the sand fraction (0.72% total carbon) (Figure 3). Both of these

TABLE 1. Particle size analysis and organic carbon contents for 3 surface soils and non-PCB contaminated Hudson River sediment.

Support	% Sand	% Silt	% Clay	% OC
Soil				
silty clay loam	15.8	56.0	28.2	1.0
clay loam	43.1	28.7	28.2	1.1
sandy loam	89.9	5.4	4.7	0.2
Sediment				
Hudson River	88.9	7.4	3.7	7.7

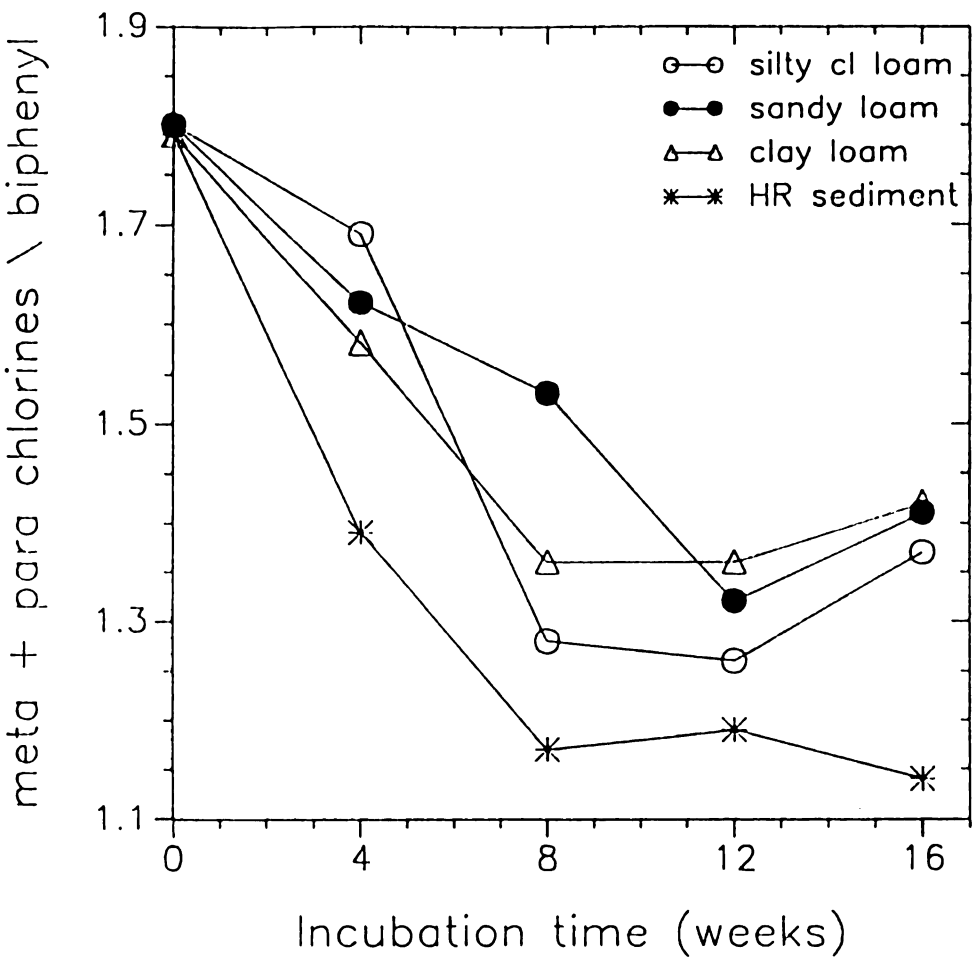

FIGURE 1. Reductive dechlorination of Aroclor 1242 by Hudson River micorrganisms in the presence of 3 Michigan soils. Data are means of duplicate samples.

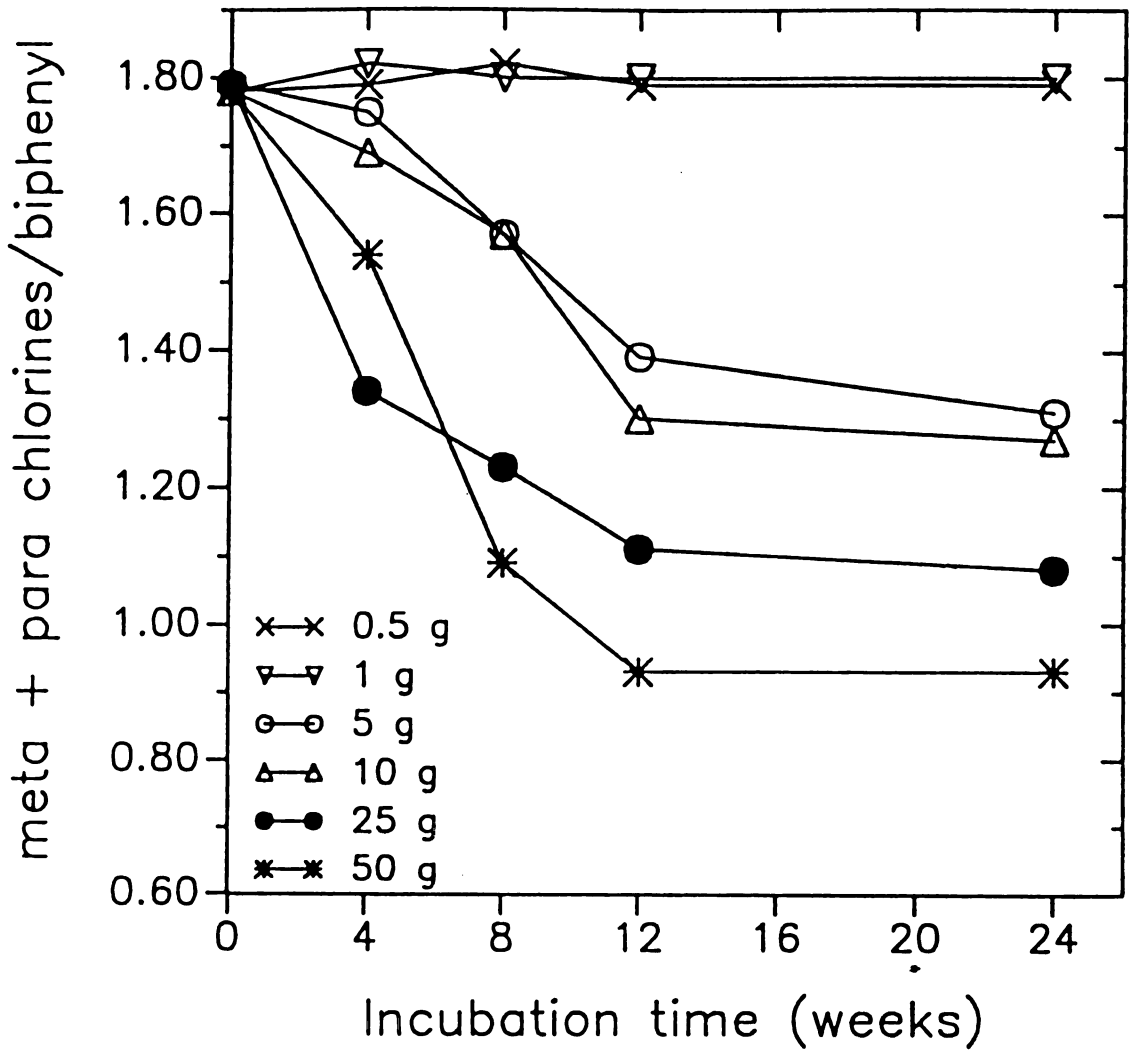

FIGURE 2. Reductive dechlorination of Aroclor 1242 by Hudson River microorganisms in the presence of varying amounts of non-PCB contaminated Hudson River sediments. Data are means of duplicate samples.

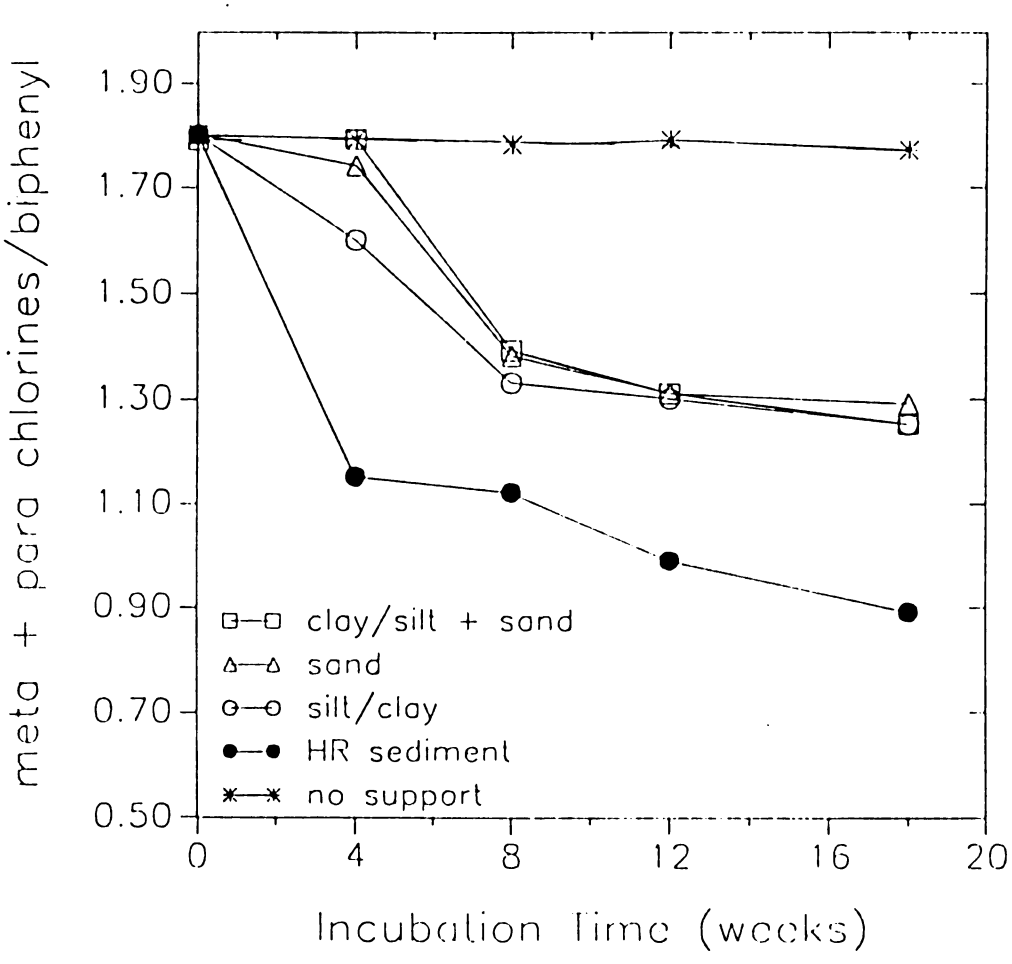

FIGURE 3. Reductive dechlorination of Aroclor 1242 by Hudson River microorganisms in the presence of fractionated non-PCB contaminated Hudson River sediment or no sediment. Data are means of duplicate samples.

fractions contain roughly the same amount of total organic carbon. The addition of 22.5 g of a clean sand (standard Ottawa, EM Industries, Inc., Cherry Hill, N.J.) to the silt plus clay fraction did not enhance the dechlorination activity. Interestingly, each of these fractions would contain 50% of the organic carbon of the intact sediment, and the amount of dechlorination these two treatments supported together would be the equivalent to the amount the intact sediment supported (each treatment removed approx. 0.4 *meta* plus *para* chlorines per biphenyl molecule).

The influence of the total carbon present in clean Hudson River sediment was studied by assaying for PCB dechlorination in the presence of ashed Hudson River sediments (Figure 4). Ashed sediment alone did not support dechlorination. However, ashed sediments with a 20% ruminal fluid or Aldrich humic acid (.04 g/serum bottle) addition supported dechlorination activity, although not to the same extent as clean Hudson River sediments. A 20% ruminal fluid medium or Aldrich humic acid did not support dechlorination in the absence of a solid suppport. The use of commercial humic acids (and Aldrich humic acid specifically) to evaluate the reactivity and role of natural humic substances has been reviewed (Malcolm and MacCarthy, 1986). The two treatments which supported dechlorination, ashed sediments plus ruminal fluid, and ashed sediments plus Aldrich humic acid, exhibited similar dechlorination patterns (predominately *meta* dechlorination) (Figure 5).

A number of alternate solid supports were tested that did not support reductive dechlorination by the Hudson River microorganisms: activated carbon, chromosorb, chromosorb plus Aldrich humic acid, chromosorb plus 20% ruminal fluid medium, Aldrich humic acid plus sand, ruminal fluid medium plus sand, Hudson River humic material plus ashed sediment, ashed sediment plus activated carbon, vermiculite, vermiculite plus 20% ruminal fluid medium, and vermiculite plus Adrich humic acid.

Abramowicz et al. (1989) studied the influences of modifications to RAMM on dechlorination of PCBs. Water alone, or RAMM minus the trace metals inhibited dechlorination activity. The addition of a complex organic extract to RAMM (e.g., FTMBE) or 1.5 times the RAMM concentration normally used enhanced dechlorination activity. This work suggest that the metal component of the reduced anaerobic mineral medium is important.

In another previous study (Griffith et al., 1990), several alternative solid supports (e.g., sand, sawdust, clay, expanded vermiculite, or clay alone) did not support reductive dechlorination. In these experiments, only anaerobic mineral medium (RAMM) was added, and a carbon source was not supplied. The treatments which supported dechlorination included peat plus expanded vermiculite, peat plus clay, and peat alone. Only the peat plus vermiculite treatment supported dechlorination of Aroclor 1242 at rates similar to clean Hudson River sediments. Treatments of peat plus clay and peat alone removed approximately half the number of chlorines compared to the peat plus vermiculite and clean Hudson River sediment treatments.

Interestingly, the PCB dechlorination patterns observed in the presence of these alternative supports differed, with the peat treatment yielding predominately *para* dechlorination, the peat plus clay mixture predominately *meta* dechlorination, while the Hudson River sediments and peat plus vermiculite treatment yielded *meta* plus *para* dechlorination. This suggest that vermiculite may serve as a good solid support, provided that all nutritional requirements are supplied to the microorganisms. In the present study, neither Aldrich humic acid or ruminal fluid with vermiculite supported reductive dechlorination. According to these results, and those of Griffith et al. (1990), vermiculite amended with peat extracts would be worthwhile to try.

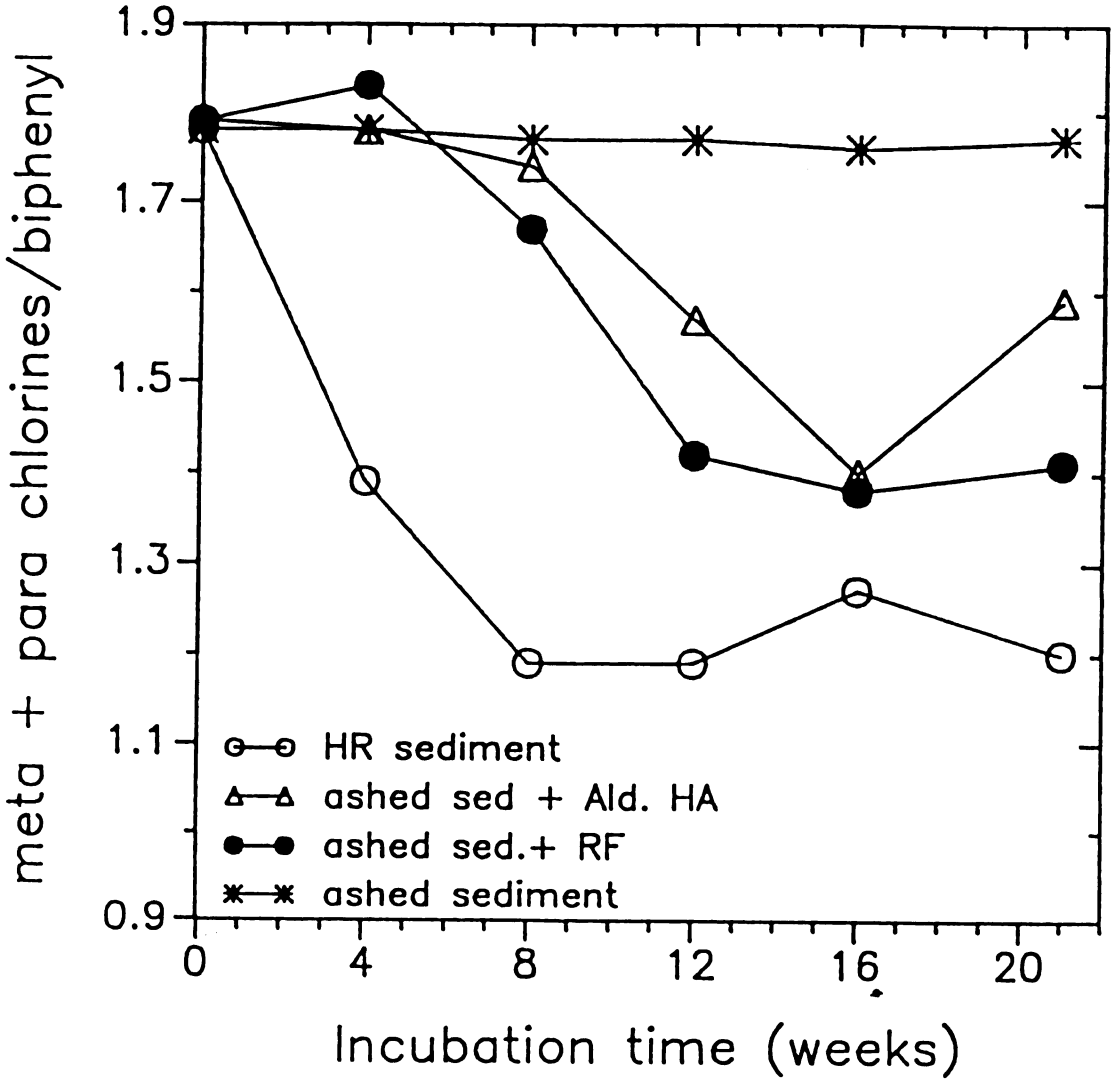

FIGURE 4. Reductive dechlorination of Aroclor 1242 by Hudson River microorganisms in the presence of ashed sediments and ashed sediments amended with Aldrich humic acid (Ald. HA) and a 20% ruminal fluid medium (RF). Data are means of duplicate samples.

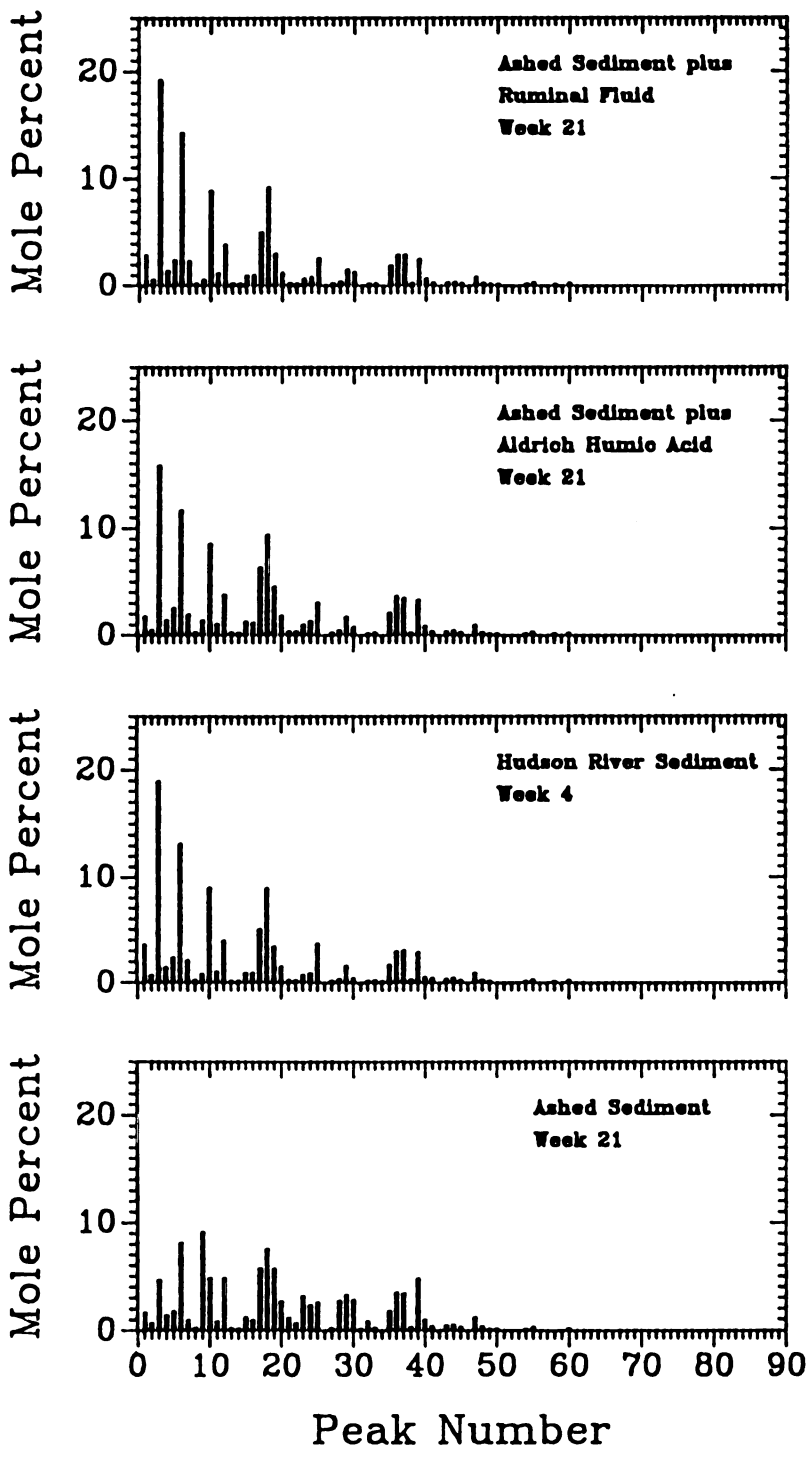

FIGURE 5. Mole percentage of PCBs (Aroclor 1242) represented by each chromatographic peak after 4 or 21 weeks of incubation with Hudson River microorganisms in the presence of ashed sediments and amended ashed sediments.

carbon, vermiculite, vermiculite plus 20% ruminal fluid medium, and vermiculite plus Adrich humic acid.

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